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CHROMATOGRAPHIC REVIEWS

Volume 3

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ELECTROPHORESIS AND RELATED METHODS

Volume 3

Edited by

MICHAEL LEDERER

Istituto di Chimica Generale ed Inorganica,
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PREFACE

The third volume of *Chromatographic Reviews* differs from the previous ones in that two hitherto unpublished reviews are included. These are the review of inorganic paper electrophoresis by R. A. Bailey and L. Yaffe and the progress report on inorganic paper chromatography by the editor.

Three other papers are continuations of reviews which appeared in the first and second volumes: Starch Electrophoresis by H. Bloemendal (Vol. 2) and The Chromatography of Phenol Derivatives by L. Reio (Vol. 1). The latter is an account of original work by the author.

The only translated review in this volume is that on Continuous Electrophoresis and Two-dimensional Electrochromatography by Z. Pučar (originally in German).

It is intended that the series of *Chromatographic Reviews* should be continued year by year so that eventually the whole field of chromatography and electrophoresis will be comprehensively covered.

Rome, January 1961 — Michael Lederer

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MULTIPLE ZONES AND SPOTS IN CHROMATOGRAPHY

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1. INTRODUCTION

The tendency among workers employing chromatography to accept two or more zones on a column or spots on a paper as indicative of heterogeneity has almost led to some erroneous conclusions. The careful work of SMITH[1] AND OVENSTON[2] which detected the anomalies in their particular investigations responsible for what has become known as multiple or double zoning or spotting is deserving of praise. It is difficult to say how many "new compounds" have been reported because of this phenomenon. It is now recognized that a single pure substance may lead to the formation of more than one zone or spot[3]. Multi-zoning is particularly dangerous with mixed chromatograms where an unknown and a known compound are mixed and chromatographed. If two spots appear the compounds are assumed to be different which may not be the case at all. OVENSTON[2] advises that the unknown and suspected known be chromatographed side by side so double spotting, if it occurs, will be obvious. As OVENSTON remarks, theoretically a single substance should give a single zone but actual chromatographic systems may be far more complex than the assumed theoretical model. Explanations may be given for particular cases but no one seems to apply to all[4]. It is the purpose of this communication to review, summarize, and perhaps extend some of these explanations.

2. SOURCES OF MULTI-ZONING

Chemical reaction

Some solutes may, unknown to the investigator, undergo chemical conversion from one form, or species, to another prior to application to the chromatogram, after application but before development, or during the course of development. Cases of reaction on active adsorbents have been known for some time[5]. Alumina is notorious in this regard. This problem and some possible mechanisms for the action have been extensively discussed in an interesting manner by MEUNIER AND VINET[6]. If the rate of A going to B is finite but the reverse reaction is very slow, or does not occur at all, then in the course of development of A, there should be two contiguous zones, one of A and the other of B if the two compounds have different mobilities. The time

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interval where the two coexist becomes shorter the faster the reaction. Rechromatography should detect this situation. In the same system, B should give one zone while A should still give two. Eventually B should be the only compound detectable.

If the immobile phase is nonparticipative in the conversion, the reaction may be oxidative or photosensitive[7,8].

Reactions that occur before development is started lead to multiple spots. BAYLY, BOURNE AND STACEY[9] found that glucose chromatographed in the presence of some ammonium salts gave three distinct, well separated spots. They later reported[10] that one of these was due to glucosylamine and another was diglucosylamine which were formed when the solution of glucose containing the ammonium salts was applied to the paper and dried in a stream of warm air prior to development. These artifacts were not formed if the origin was dried in cool air. Since the reaction attained equilibrium before development, the spots were distinct. These experiments were repeated for mannose, glucose, and galactose by RAACKE-FELS[11] who proposed for mannose a series of reversible equilibria between mannose, mannose–ammonia, mannosylamine, and dimannosylamine. The reversible reactions of mannose–ammonia proceed at rates which do not permit resolution on the chromatogram. The author proposes that the reaction is between the sugar and ammonia rather than ammonium salts, the latter being catalysts. Also, heat is apparently not necessary.

Impurities in the solution to be chromatographed

STRAIN[12] has pointed out that extracts from plants containing pigments many times contain colorless components which lead to odd results. The inexperienced researcher, intent upon the pigments, may inadvertently perform some manipulations which lead to some unusual performances of the materials and to false conclusions. These adsorbed, colorless impurities may act as displacers during development. A solution of 2 to 3 mg of lutein or zeaxanthin per 100 ml of petroleum ether–25 % acetone and containing a small amount of a strongly adsorbed material, *e.g.*, propylene glycol, when developed with the petroleum ether–acetone solvent on a magnesia column gave a dark leading zone followed by a second faint zone. The two zones appeared separated by a zone devoid of pigment. A weakly adsorbed contaminant can give a faint leading zone and a contiguous dark second zone. In both cases, prolonged development gives a single zone.

The addition of a contaminated pigment solution to a column followed by a second, uncontaminated solution of the same pigment in the same solvent may lead to two zones which later recombine on prolonged development. If the two pigment solutions are mixed before addition to the column, one zone is produced. This observation should be a warning to those who combine extracts on a column.

The appearance of a double zone is given when the initial zone contains several fold the amount of material that can be dissolved in the developer occupying this zone (overloading). The solute may be regarded as "precipitated" relative to the developer. It is dissolved at the tail of the initial dark zone, is moved through the band and appears as a faint zone, leading the initial zone and contiguous with it.

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Band inversions may temporarily give the false impression of multiple zoning. If A is above B when a solution of the two in solvent 1 is placed on the column and if the order is reversed with adsorption from solution in solvent 2, then on formation of the chromatogram with solvent 1 and development with solvent 2, A will migrate through B and one zone appears to be present during the inversion. Prolonged development gives two zones.

Discontinuities in the immobile phase

Columns of kieselguhr treated with a liquid are generally accepted as the clearest example of liquid–liquid partition chromatograms[13]. The immobile liquid phase seems to consist of micro-drops dispersed through the inert support, where it is loosely held and subject to change particularly with developers which are not exactly saturated with the immobile phase. SMITH[1] chromatographed the B vitamins on kieselguhr holding one-half its weight of water or dilute buffers suspended in water-saturated *n*-butanol and developed with water-saturated *n*-butanol. If a portion of *n*-butanol containing excess water dispersed as colloidal droplets is passed through the column, the kieselguhr removes the water to give a locally continuous wet phase at the top of the column which could trap a portion of the solute. If this is developed with water-saturated *n*-butanol, the untrapped solute is washed from this region to give a band lower down on the column and leaving a band at the top. Since the immobile phase no longer exists as small droplets, equilibrium could not be established with the mobile phase and the solute is only slowly leached from the upper portion. This leads to two bands joined by a very pale region. If the column is first treated with butanol containing less than the saturation amount of water, immobile phase may be removed from the top portion of the column to give a "dry" region which can lead to double zoning on development with water-saturated *n*-butanol. Since the solubility of water in butanol is strongly temperature-dependent, these same discontinuities can be produced by a temperature variation of as little as 5° during chromatography.

OVENSTON[2] had a similar experience with silica-"Celite" columns whose adsorptive properties also depend on water content. When the column was prewashed with diethyl ether, the ether partially eluted water from the column. When the ether wash was stopped before the excess water was completely removed and the chromatogram formed and developed with a different solvent of weaker eluting power, an intermediate wet zone was formed on the column which led to double zoning at the discontinuity. Petroleum ether will remove the ethyl ether without affecting the wet zone. The two solute zones, once passed the discontinuity, will eventually reunite in this case. However, if the developer also causes the wet zone to migrate at a rate comparable to the adsorbate, they may not reunite. OVENSTON warns that double zoning is likely to occur if the column is first treated with a strong developer either in a prewashing treatment or during chromatography and is followed by a weaker developer. It would seem preferable to prewash adsorbents by decantation before packing the column to avoid such discontinuities.

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A similar explanation may apply to the double zoning observed by SCHROEDER[4] on silicic acid columns.

Large amounts of inorganic salts in the solution applied to the chromatogram may produce water-logging near the origin by a salting out effect[14].

Discontinuities in the mobile phase

BOMAN[15] applied human serum albumin along an oblique line on a sheet of paper and developed the chromatogram with ethanol–phosphate buffer. The final spots should also have been on an oblique line. However, they appeared on a horizontal line. The explanation given is that the albumin travels close to the developer front and during its journey down the paper is partly adsorbed by the paper. It is soon all adsorbed to give a series of narrow elongated spots nearly parallel to the initial oblique line. The developer front appears to be followed by a "second front" which causes some change in the paper, possibly in electrical charge, which releases the albumin which then travels with this front. This front, parallel to the first, aligns the spots on a horizontal line. In certain cases, the solute appears as a double spot, one between the first and second front and one at the second front. No real discontinuity need exist in the mobile phase. If some sort of gradient of some property exists in the developer in the direction of flow and if at some particular value of this property the albumin is released, then the chromatogram gives the appearance of two fronts in the mobile phase. This sudden change of adsorbate may be pictured as similar to the sudden change in pH at the end point when a strong acid is slowly added to a strong base in the course of a titration. NUNES DA COSTA AND GUEDES DE CARVALHO[16] have observed double zoning accompanying double fronting in the paper chromatography of the borates.

Charged species and complexes

Whenever a solute can exist as one or more uncharged species and/or as one or more charged species, or is capable of forming complexes, multi-zoning can occur. This is probably the least understood cause of multiple spots.

LANDUA, FUERST AND AWAPARA[17] found that tailing and double spotting of amino acids depended strongly on the pH of the solution applied to the paper. All compounds were developed with phenol–water. The observation that the solutes could exist as one or more species and that double spots were always connected by a diffuse region is important as will be shown later. ARONOFF[18] found the same situation for lysine and that the relative intensities of the spots were in rough agreement with the distribution of ionic species as calculated from the pH of the solution applied. The lysine ions would be capable of association with the phenol to give new compounds with their own partition coefficients. WESTALL[19] has proposed a similar association to explain the separation of sodium and chloride ions on paper when developed with phenol–water. The sodium ions supposedly form a phenate which is more soluble in the phenol and shows a higher R_F.

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Various dissociations may be visualized. If the solute is capable of accepting a proton

$$A + H^+ = HA^+$$

the ion formed may associate with phenate ion

$$HA^+ + C_6H_5O^- = AHOC_6H_5$$

Ionic species would be retained by the polar non-mobile aqueous phase while the unionized compound formed with the phenol would be more soluble in the mobile phase and migrate with a higher R_F. The initial distribution of charged and uncharged species would be fixed by the pH of the solution applied. If development disturbs the acid concentration, and it is difficult to see why it shouldn't since the pH of the developer is not adjusted by a buffer, the equilibria should readjust to that given by the solute in phenol–water and the chromatogram obtained should be the same in all cases, that is, independent of the initial pH. The fact that this is not true indicates a more complicated change. As will be shown, definite discrete double spots will be obtained only if the rate of conversion is slow compared to the time of development of the chromatogram. If the rate of conversion is fast compared to the time of development, one spot should be obtained. Between these two extremes, serious tailing and multiple spots joined by diffuse regions may occur. This argument, of course, makes the assumption that in the earlier stages of development, the hydrogen ion causing the initial distribution is removed from the origin and that the system is either irreversible or attempts to attain the distribution it would have in the developer. That the first part of this assumption is questionable is shown by WALDRON-EDWARD[14] mentioned below.

The rate of interconversion of charged and uncharged species is not the only factor, however. CURRY[20] observed multiple spots when he chromatographed the different acid phosphate salts in pyridine–ethyl acetate–water. The number and intensities of the spots depended upon the pH. If orthophosphoric acid is used, the faster spot predominates; if the normal salt of this acid is used, the slower spot predominates. He assumed that the spots were due to $H_2PO_4^-$, HPO_4^{-2} and PO_4^{-3} and that pyridine complexes were not involved since similar results were obtained with butanol. When orthophosphoric acid with radioactive phosphorus was used, the activity was 6000 counts/min at the spots but only 100 counts/min between spots showing the spots to be well resolved. This could be explained by a slow proton transfer

$$H_2PO_4^- = HPO_4^{-2} + H^+$$

To test this he chromatographed a mixture of labeled $H_3P^*O_4$ which ought to give $H_2P^*O_4^-$ and unlabeled Na_2HPO_4 which ought to give nonradioactive HPO_4^{-2}. Both final spots obtained were radioactive indicating an appreciable rate of proton transfer. A close examination of CURRY's paper indicates that the tagged and untagged species were mixed before application to the chromatogram. This would conceivably permit proton transfer before chromatography and one would expect activity in both spots. He did not feel that multispotting was due to the interference of inorganic

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ions or impurities in the paper since the paper was acid-washed which generally is the accepted procedure for removal of such impurities. Multispotting was observed with 2′,3′-isopropylidene-adenosine-5′-phosphate. ERDEM's[21] chromatograms with arsenates were very similar to CURRY's. ERDEM also obtained two to three distinct spots with divalent cadmium in the presence of ammonia. All of the spots contained cadmium and were attributed to different amino complexes. ERDEM AND ERLENMEYER[22] point out that one must assume that the kinetics of the transformation are radically different in the immobile phase than in the mobile phase. They cite examples from the literature of adsorption as justification. Their assumption requires that paper chromatography involves an adsorption mechanism rather than a partition between an immobile liquid-gel and a mobile fluid as proposed by MARTIN[23] and discussed by MOORE AND STEIN[13]. MARTIN[24] suggests that reaction rates in the liquid-gel do not differ much if at all from those observed in the pure liquid. The suggestion of radically different reaction rates in the immobile phase in paper chromatography must be regarded with suspicion unless more supporting evidence is found.

Impurities in the paper may very well cause unusual results. HANES AND ISHERWOOD[25] working with inorganic phosphate, traced "ghost spots", or retention of some of the solute at the point of application, to the presence of calcium and magnesium ions in the paper. This was eliminated by prewashing the paper with acid. These ions are also responsible for "shadows", faint regions following the principle spot but different from tailing. Multiple banded spots, which give the appearance of a well defined dark spot overlapping a fainter but equally well defined second spot, could be overcome by a strong organic acid in the developer.

WALDRON-EDWARD[14] found that D-glucosamine gave two spots when sulfate ion was present in the solution applied to the chromatogram in an amount equivalent to the amine; that the slower spot only was produced with excess sulfate and that the second spot always contained sulfate which was not present anywhere else in the developed paper. Similar effects were found with lysine, arginine, ornithine, histidine, ethanolamine, tyramine, ethylene diamine, and tetramethylene diamine. Some of these same substances were found to give unusual behavior by LANDUA, FUERST AND AWAPARA[17] and by MCFARREN[26] who investigated amino acid chromatography on buffered papers.

Earlier it was assumed that the developer separated the solutes from the inorganic ions of the buffer in the solution applied to the chromatogram thus disturbing the equilibrium. If these ions migrate with the solutes, however, they may maintain the equilibrium or the position of equilibrium may be changed only slowly by their gradual removal. Such a possibility increases the complexity of the problem. Not only are rates of conversion important but also the position of the equilibrium. The latter may be fixed by a buffered paper which may partially account for the success of this technique.

The problem of inorganic salts and their role in salting out substances, forming complexes, and migrating with developer is a formidable one. If one adds to this their possible participation in an ion exchange mechanism[27] the situation becomes worse.

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Equilibrium between two species

Possibly one of the best examples of reaction between two forms of the same solute and which is free of disturbing influences such as inorganic ions is given by PARTRIDGE AND WESTALL[28] who chromatographed the sugars and related compounds on paper using phenol, *s*-collidine, and *n*-butanol–acetic acid. Neutral reducing sugars gave well defined spots indicating that the tautomeric equilibria known to occur in water did not affect the chromatogram. In butanol–acetic acid a mixture of glucuronic acid and its lactone, glucurone, gave two well separated spots; glucuronic acid gave two well defined and unjoined spots while glucurone gave a very distinct lower spot and a smaller, fainter, well separated slow spot. In *s*-collidine, glucuronic acid gave a dark slow spot and a smaller, fainter faster spot; glucurone gave two spots joined by material all the way along the chromatogram. In general, the leading spot showed tailing while the slow spot showed bearding. This observation is in accord with theory. If a substance exists in two forms which are interconverting and if the rate of conversion is slow relative to the time of development, then two spots can be obtained. There would be material joining these spots all the way along the chromatogram but its detection depends on the sensitivity of the method of revelation. The lead zone should show tailing while the slow zone should show bearding.

Theoretical treatments of differential migration have been given for sedimentation and electrophoresis[29-31]. If one accepts that these processes all represent the same type of phenomena, that is, differential migration of substances in a phase when subjected to some driving force, then these results should be qualitatively adaptable to chromatography. We will outline, in detail, a method for calculating concentration profiles for general differential migration methods and a method for calculating spot shapes in paper chromatograms.

3. INTERCONVERTING SPECIES

It is a little known fact that a large class of chromatographic anomalies can appear as a result of an involved kinetics. It is a general requirement that one of the kinetic steps be slow so that the system is not near equilibrium. The kinetic scheme discussed below, which is just one example of many possible schemes, leads to the formation of tails, comets, beards, double spots, and even triple spots, with the appropriate choice of parameters.

Suppose that a substance can exist in two different, interconvertible forms, A and B, each with a degree of permanence shown by a slow rate of conversion between them. Suppose, also, that each form undergoes the usual chromatographic exchange (adsorption or partition) between the mobile and immobile phases and back again. This exchange is denoted by $A \rightleftharpoons A'$ where A designates a molecule of the species A in the mobile phase and A′ designates a molecule of this species in the immobile

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phase. A similar expression can be written for species B, *i.e.*, B $\rightleftharpoons$ B′. Both of these exchanges are assumed to proceed rapidly. The net scheme is

The slow reaction between A and B proceeds with first order rate constants, k_1 and k_2.

Zero rate constants

The investigation of the above scheme can be best approached by first assuming $k_1 = k_2 = 0$, *i.e.*, with no interconversion between A and B. Each species will migrate independently of the other and the zone of each will broaden during migration. This broadening is due to the combined result of the partitioning kinetics, molecular diffusion, and "eddy" diffusion[32,33]. Each of these phenomena can be assigned an apparent diffusion coefficient to describe its contribution and the sum of these gives an effective diffusion coefficient D which describes the spreading of the zone. When D itself is variable (due to non-constant flow rates, etc.) then a time average of D must be used. If the initial zones are very narrow bands and a diffusion model describes the spreading, then the concentration profiles, at any later time during development are Gaussian in shape.

The picture then, with zero rate constants, is of two zones migrating with their own R_F's and each spreading according to their individual values of the net diffusion coefficient D. If the mobilities of A and B are sufficiently different and zone broadening is not too great, well defined and separated spots should result. This case corresponds to the observations of Bayly, Bourne and Stacey[9], and Raacke-Fels[11].

This picture is modified for the reversible conversion of A to B with finite transition rates. The following simple argument shows the nature of the new concentration profiles. Any molecule that was originally A and has changed to B or *vice versa*, has spent part of its time migrating with an R_F characteristic of A, and the remainder of its time with the R_F of B. Hence at a later time, t, such a molecule will be found somewhere between the A and B zones at the two extremes. Thus there will appear two zones or spots with a diffuse streak between them.

The question next arises as to the amount of material in the two extreme zones relative to the material between them. Since the reactions are all first order, the end zones disappear by exponential decay. If the original material is divided with the fractions α and β in the A and B forms respectively, then at the later time t, $\alpha \exp(-k_1 t)$ will be the fraction in the A form and $\beta \exp(-k_2 t)$ will be the fraction in the B form. The total fraction of material in the intermediate streak will be $1 - \alpha \exp(-k_1 t) - \beta \exp(-k_2 t)$. At sufficiently long times, compared to both $1/k_1$ and $1/k_2$, this will approach unity and essentially all the molecules will be somewhere between the expected positions for A and B with no interconversion. Under the condition of large t, nearly every molecule will have made several A $\rightleftharpoons$ B transitions, and the concentration profile will approach a Gaussian distribution with the peak somewhere in the

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center, its position depending on the relative average times spent in the A and B forms.

A quantitative evaluation of the concentration profile can be made using chromatographic theory[33a]. In its simplest form, this theory gives the probability distribution for the relative times spent in the A and B forms when the reaction scheme is, as above, A $\rightleftharpoons$ B. If we let the fraction of time that a molecule spends as A be x, then the probability that this fraction is in the range x to $x + dx$ is

$$P_1(x)\,dx = a \exp\left[-a\,(1-x) - bx\right] I_0 \sqrt{4abx\,(1-x)}\,dx \qquad (1)$$

providing that the molecule was in the A form at the beginning of the run and in the B form at the end. In this expression, $a = k_1 t$ and $b = k_2 t$. I_0 is a Bessel function of imaginary argument.

If the molecule starts out as A and after at least one reaction cycle, ends as A, then the probability becomes

$$P_2(x)\,dx = \left[\frac{ab\,(1-x)}{x}\right]^{1/2} \exp\left[-a\,(1-x) - bx\right] I_1 \sqrt{4abx\,(1-x)}\,dx \qquad (2)$$

The only other possibility, starting with A, is that no reaction at all occurs. The probability of this case, for which $x = 0$, is $\exp(-a)$.

The remainder of the concentration profile comes from the molecules that are originally in the B form. The $P(x)$ expressions in this case are obtained from the previous equations by substituting a for b and $(1-x)$ for x. The corresponding equations, first for a molecule that begins as B and ends as A (eqn. (3)), and second for a molecule that begins as B and returns to B after one or more reaction cycles (eqn. (4)) are

$$P_3(x)\,dx = b \exp\left[-a\,(1-x) - bx\right] I_0 \sqrt{4abx\,(1-x)}\,dx \qquad (3)$$

$$P_4(x)\,dx = \left(\frac{abx}{1-x}\right)^{1/2} \exp\left[-a\,(1-x) - bx\right] I_1 \sqrt{4abx\,(1-x)}\,dx \qquad (4)$$

The probability that no reaction occurs, for which $x = 1$, is $\exp(-b)$.

The overall concentration profile is proportional to the final probability density function, $P(x)$, which is obtained by properly weighing the above expressions. Since the original fractions of molecules in the A form is α and in the B forms is β, then

$$P(x) = \alpha\left[P_1(x) + P_2(x)\right] + \beta\left[P_3(x) + P_4(x)\right] \qquad (5a)$$

$$P(x = 0) = \alpha \exp(-a) \qquad (5b)$$

$$P(x = 1) = \beta \exp(-b) \qquad (5c)$$

We have computed $P(x)$ for several different sets of the parameters a, b, α, and β. In the typical case, $P(x)$ is continuous and finite in the interval $0 < P(x) < 1$. At $x = 0$ and $x = 1$, an infinitely thin slice has an area on the normalized $P(x)$ curve of $\alpha \exp(-a)$ and $\beta \exp(-b)$. Since this cannot be shown graphically, we have included this area in a Gaussian distribution centered about $x = 0$ and $x = 1$ and with a finite standard deviation of $\sigma = 0.1$. This procedure, for a typical case, is illustrated in Fig. 1.

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The distribution of material shown by the $P(x)$ curves in these figures results only from the A $\rightleftharpoons$ B reaction and has not yet included the actual chromatographic process A $\rightleftharpoons$ A′ and B $\rightleftharpoons$ B′. This situation is easily remedied, however, since a single diffusion coefficient may be used to describe the combined effects of the chromatographic process, ordinary molecular diffusion, and eddy diffusion[32–34]. In order to calculate this, we set up a numerical program which allows for the diffusion of the material in the zone described by $P(x)$. The Schmidt method has been used for this purpose[35]. The calculations of this method can be made in less than an hour with the use of a desk calculator. We have restricted our considerations to the case where the diffusion coefficients of species A and B are the same. If it seems desirable to pursue the less restricted case where they are different, this can be easily done.

The choice of the diffusion parameters is independent of those chosen to represent the kinetics of the reversible conversion of A to B. We have kept ordinary diffusion

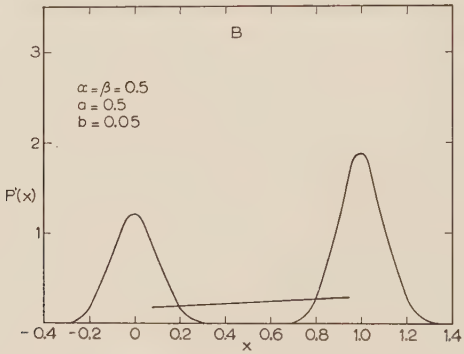

Fig. 1. The probability (concentration) density, $P(x)$.

at a minimum to emphasize the kinetic effects. In practical cases diffusion may often be a more important factor which would manifest itself simply as an additional spreading of the profile over and above that presented here. Our calculations are made with an effective diffusion coefficient

$$D = \frac{[0.1\ (R_{FB} - R_{FA})\ v]^2}{2t} \tag{6}$$

where R_{FA} and R_{FB} are the R_F values of the two species and v is the velocity of the mobile or developer phase. This value was chosen so that the root-mean-square displacement of a molecule by diffusion is one-tenth the distance between the centers of the A and B zones. The new probability density function, $P'(x)$, is obtained by adding the "diffused" $P(x)$ profiles to the Gaussian curves representing the end zones. Fig. 6 is the final result.

4. CALCULATION OF SPOT SHAPES

The $P'(x)$ profiles calculated above yield some representative concentration profiles which are useful if concentrations are measured directly as is often the case in the elution techniques. Quite often, however, zones or spots are observed visually,

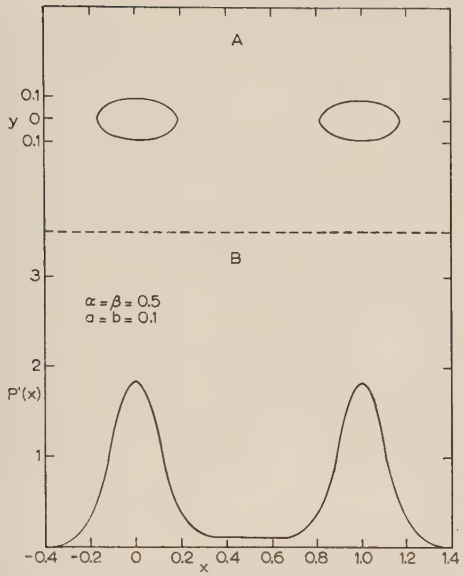

Fig. 2. Concentration profile and spot outlines for a symmetrical case. With a more sensitive method of detection, a continuous streak would exist between the two spots corresponding to the finite concentration between them.

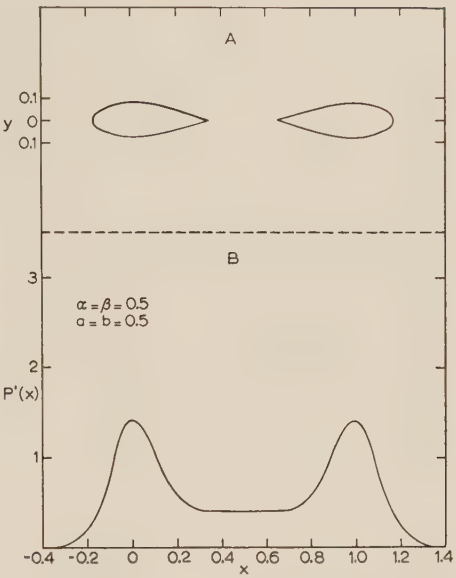

Fig. 3. A symmetrical case in which the two spots tail towards one another due to the increased concentration in the center.

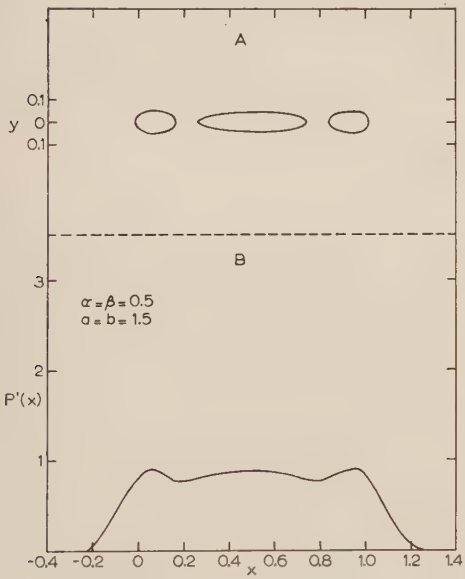

Fig. 4. Triple spots will be seen for this case if the initial spot content and the sensitivity (cutoff) fall within narrow limits. Since the minima in the concentration profile are not prominent, a streak would ordinarily appear between each spot. Otherwise the spots will be quite faint.

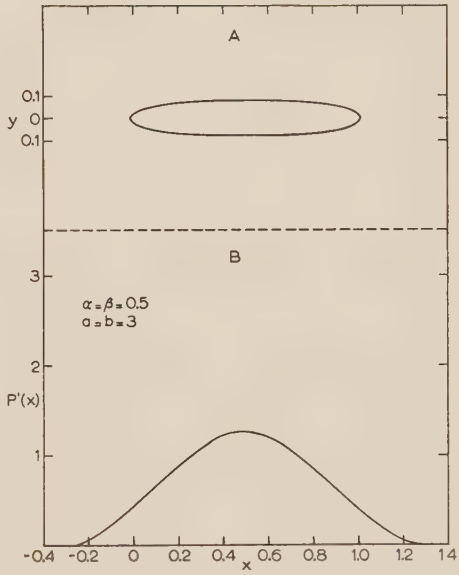

Fig. 5. Elongated spot resulting from the extended development of double spots with an origin in slow kinetic steps.

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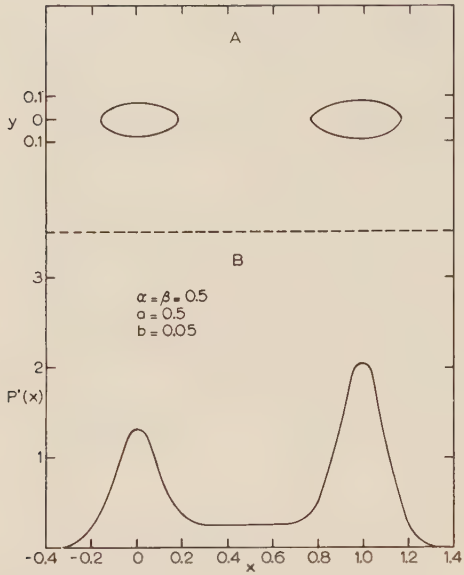

Fig. 6. Asymmetrical case. With a different spot content or cutoff this might appear as a single spot with bulging ends, double spots, or a single spot.

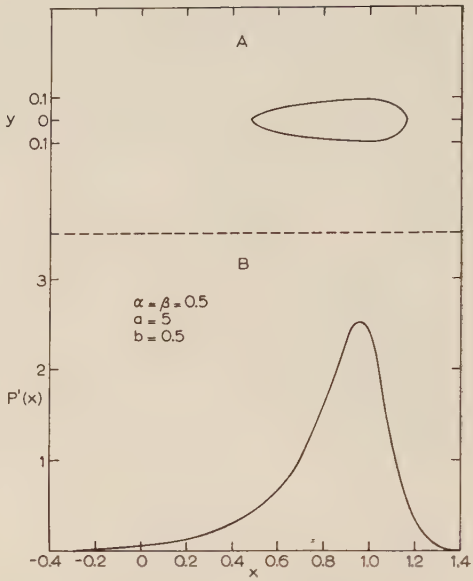

Fig. 7. Asymmetrical case in which one spot has essentially disappeared. Notice the extended tailing in the concentration profile and in the spot.

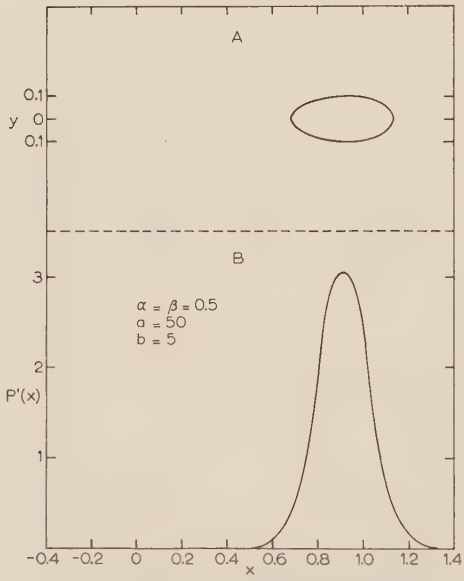

Fig. 8. Asymmetrical case with extended development. The spot appears elliptical.

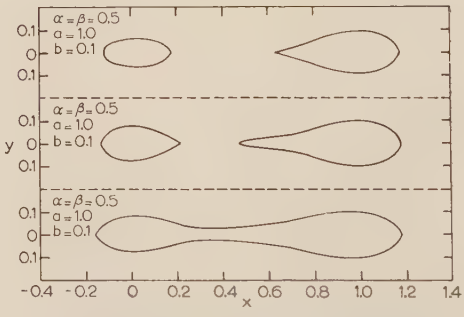

Fig. 9. Spot outlines at different cutoff values for an asymmetrical case. The range of phenomena that can be found is partially illustrated here. All the spots illustrated will, in practice, have a diffuse rather than a sharp boundary.

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sometimes with the aid of a revelation reagent. In this case it is useful to express the above theory in terms of spot sizes and shapes which are encountered in paper chromatography. In this way the researcher is provided with suggestions as to the kinetic processes contributing to his chromatographic results.

In accordance with the previous communication in this series[33] we assume that the material spreads both in the direction of developer flow (longitudinal) and perpendicular (lateral) to it with different effective diffusion coefficients for the two directions. If the width of the original spot is small the lateral distribution of material will appear as a Gaussian distribution with $\sigma_y = (2D_y t)^{1/2}$ where D_y is the effective diffusion coefficient in the lateral direction. On this planar chromatogram any material is apparent as a spot (*i.e.*, within the spot boundary) when the concentration exceeds a certain critical or cutoff value (determined by the limits of visibility, etc.). This value is obtained when the two-dimensional density function equals P_0. The following equation describes the lateral extension $y - \bar{y}$, of the spot in terms of P_0

$$P_0 = \frac{P'(x)}{\sqrt{2\pi}\sigma_y} \exp\left[-\frac{(y-\bar{y})^2}{2{\sigma_y}^2}\right] \tag{7}$$

The width of the spot is $w = 2(y - \bar{y})$ and this becomes

$$w = \sigma_y \left[8 \ln \frac{P'(x)}{\sqrt{2\pi}P_0\sigma_y}\right]^{1/2} \tag{8}$$

When w becomes an imaginary number this is to be taken as zero. Spots with widths obeying this formula have been drawn in Fig. 2 through 9 along with the $P'(x)$ profiles from which they are obtained. The cutoff point is altered by changing P_0. It should be noted that the spot might appear thicker or thinner simply by variations in σ_y.

5. DISCUSSION OF THE RESULTS

The theoretical analysis of concentration profiles is a general method of treating differential migration processes. In electrophoresis, for example, the same formulation applies simply by substituting the two velocities v_A and v_B for $R_{FA}v$ and $R_{BF}v$. Hence a substance composed of two slowly interconverting forms can appear as two zones and sometimes as three zones, in either electrophoresis or chromatography.

The problem of zone distribution in electrophoresis has been treated by a number of authors. CANN, KIRKWOOD AND BROWN[31] set up the one-dimensional differential equations for the distribution of two interconverting species undergoing electrophoretic migration. They were able to obtain a solution only for a limited set of parameters, but they did observe double zoning in several cases. This method seems more difficult than the present one for this type of calculation since they indicate the need of a computer to extend the range of their parameters.

The cases of zone and spot distribution that we have examined are necessarily limited. There are so many physical parameters entering the calculation that it is impossible to explore the entire range of them in a single writing. The group we have

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examined is divided into two categories. In Figs. 2 through 5 we have treated the symmetrical cases where $a = b$ and $\alpha = \beta$. Asymmetrical cases are treated in Figs. 6 through 9 where $a \neq b$ and $\alpha = \beta$.

In the symmetrical cases we have shown examples where a and b successively increase. This roughly represents the calculation of a given chromatogram at successively longer time intervals. Since our profiles are plotted against the reduced variable, x, the end zones do not move farther apart with increasing time as they do in practice. It should be noticed that the principle result of increasing a and b is that of reducing the end zones and filling in the region between them. This has proceeded far enough when $a = b = 1.5$ that there appear three peaks, the center one being comparable in size to the end ones. Circumstances favorable to the appearance of three peaks are not often found since the peaks, when they appear simultaneously, are never very prominent.Three peaks would be more commonly observed with three slowly reacting species.

For larger values of a and b the end zones rapidly become insignificant. Thus where $a = b = 3$ they are swamped by the larger amount of material in the center. The corresponding zone is not Gaussian, however, until a and b become larger.

The parameters for the asymmetrical cases have been chosen arbitrarily such that $a = 10\ b$. Thus while an equal amount of material starts in each zone, the A zone is depleted more rapidly. The results show an asymmetry that is very similar to cases where $\alpha \neq \beta$. Whether the asymmetry is caused by an inequality in initial distribution ($\alpha \neq \beta$) or by an inequality in rates ($a \neq b$), or both, can be determined by further development of the chromatogram. The further depletion of material from the end zones depends directly on a and b, and if these are equal, the fractional depletion in a given time is always the same.

When a and b are unequal, as for the cases calculated, the relative depletions are unequal. Thus with $a = 10\ b$, the A zone disappears more rapidly than the B zone. This is seen in the successive Figs. 6 through 8.

With each of the Figs. 2 through 8, we have shown at least one spot outline. The encircling line represents the limit of detection around the edge of the spot. Since this limit varies with the method of detection, we have shown some examples where the concentration profile is the same but the spots differ because this limit, or cutoff, has been changed, or because the spot content is different.

With symmetrical parameters the spots are distributed equally on each side of the point $x = 0.5$. While in practice such a symmetrical distribution is not to be often expected, the calculations provide some useful prototypes. In the succession of examples we see two separate elliptical spots, two spots that are elongated towards their common center (with a different cutoff, this would become a thin streak between them), a case where triple spots are to be expected, and finally a single spot, nearly elliptical, indicating the accumulation of most of the material in the center region. This series illustrates the general tendency of two zones to merge into one on prolonged development.

The series of spots obtained with asymmetrical parameters show a similar trend.

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It is noticeable that the smaller spot is more nearly elliptical while the larger spot tends to trail off in the direction of the smaller one. In the last two figures the material is nearly depleted from the left hand side and no spot appears there. Notice in Fig. 7 the definite appearance of tailing in the spot. Tailing can appear under a variety of circumstances, even when the isotherm and kinetics are linear.

The ideal spots shown here are not always closely reproduced experimentally. A number of spot imperfections, such as missing corners and bulges, can be attributed to the heterogeneity of the paper. The heterogeneous nature of flow in paper often displays itself in the uneven shape of the solvent front.

While we have computed a representative sample of spot shapes in chromatography, the meaning of the results should not be misconstrued. We have used sets of physical parameters that are certainly not unique in providing the resultant spots. An observed set of spots may have its origin in:

1. The theory outlined here with the physical constants used here to obtain a similar set.

2. The theory used here but with a different set of constants.

3. An entirely different theoretical basis as outlined in the earlier parts of this paper. In order to decide between the three possibilities a careful study of the system must, in all cases, be made. The attempt here, has been simply to show that an entire group of phenomena will often appear as a result of the intrinsic kinetics of the system.

TABLE OF SYMBOLS

k_1	rate constant for A $\rightarrow$ B interconversion
k_2	rate constant for B $\rightarrow$ A interconversion
D	effective coefficient of longitudinal diffusion
α	fraction of material originally in the A form
β	fraction of material originally in the B form
t	time
x	fraction of time that a molecule spends in A form
a	$k_1 t$
b	$k_2 t$
$P_1(x)$, $P_2(x)$, $P_3(x)$, $P_4(x)$	probability densities given in equations (1–4)
I_0, I_1	Bessel functions
$P(x)$	overall probability density before diffusion
$P'(x)$	overall probability density after diffusion
P_0	cutoff probability density in two dimensions
σ, σ_y	standard deviation due to diffusion
D_y	effective coefficient of lateral diffusions
P_y	lateral coordinate
w	width of spot
v_A, v_B	velocities of A and B
R_{FA}, R_{FB}	R_F values for A and B

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ACKNOWLEDGEMENTS

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6. SUMMARY

Cases in which multiple spots appear in the chromatography of single substances are given. The various theoretical interpretations of multiple spotting are discussed and related to one another. Theoretical equations are derived for the evaluation of concentration profiles and spot shapes when the origin of multiple spotting is the slow interconversion of chemical species. A number of examples which give typical spot profiles are shown. Methods for determining the actual origin of multiple spotting are discussed.

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STARCH ELECTROPHORESIS

II. STARCH COLUMN ELECTROPHORESIS

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1. INTRODUCTION

In the first part of this survey on starch electrophoresis[63] the starch block technique was discussed and the conclusion was drawn that generally the best separations are realised when the starch block is in a vertical position during the electrophoretic run. In principle similar results are obtained when columns are used. Nevertheless I wish to make a distinction between vertical starch block electrophoresis and starch column electrophoresis, because the analytical operations after the experiment are different in these two methods. In the first technique the block is cut into equal segments to separate the zones, whereas in the second the separated zones are forced out by means of a buffer stream, which makes the use of a fraction collector necessary. Although originally only starch was used as the inert supporting medium[3,4] in column electrophoresis, at present there is a tendency to apply cellulose powders instead of starch[61–69]. Actually more separations are carried out with other media than starch as stationary phase. These experiments will not be discussed in this article. But even cellulose column electrophoresis is not so widely applied as starch block electrophoresis and starch gel electrophoresis. Undoubtedly the reason for this is that in general no better results are obtained with column electrophoresis than with the two last-mentioned techniques, while its procedure is more complicated.

2. METHODS

Apparatus

HAGLUND AND TISELIUS[70] described an apparatus for glass powder electrophoresis, which consisted mainly of a U tube and two electrode vessels. This apparatus was improved by FLODIN AND PORATH[4] who replaced the glass powder by potato starch.

A glass tube s (Fig. 7), 50 × 3 cm, with a sintered glass filter f at the bottom is joined to a second tube by means of rubber tubing. The porosity and thickness of the glass filter must be chosen so that the electrical resistance is as small as possible. The filter surface may be covered with a disc of Whatman No. 1 paper. The tube, which is filled with starch, is connected to the cathode and the second tube is connected to the anode, both connections being made with rubber tubing that can be closed by clamps. Reversible silver–silver chloride electrodes as well as platinum wire wound round glass rods may be used as cathode and anode. HAGLUND AND TISELIUS recom-

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mend the use of an open system, which allows the electroosmotic flow to develop freely. This is realized by connecting a narrow horizontal glass tube, open at one point, to the electrode vessels. In this way a constant hydrostatic pressure is maintained in the whole system during the electrophoretic run. In some experiments the apparatus is placed in a thermostat.

VANDEGAER, PRÉAUX AND LONTIE[71] found a more simple solution for the cooling problem. The apparatus that these investigators employed was in principle the same

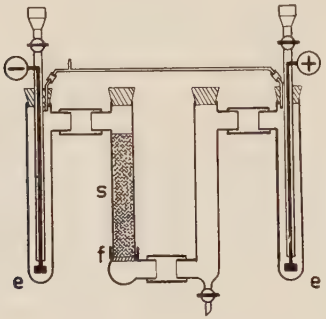

Fig. 7. Apparatus according to HAGLUND AND TISELIUS. e = electrodes; s = tube filled with starch; f = glass filter.

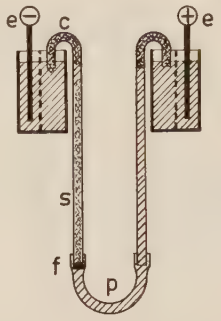

Fig. 8. Apparatus according to KUNKEL. e = electrodes; s = tube filled with starch; f = filter disc; c = cotton wicks; p = plastic tubing.

as that of FLODIN AND PORATH, but they constructed a water-jacketed column. Efficient cooling was obtained by tap water of 2°.

CARLSON[3], keeping to the design of HAGLUND AND TISELIUS, placed a central cooling tube in the starch column.

BOLINGER, VAN DER GELD, WILLEBRANDS AND GROEN[72] improved the original apparatus of FLODIN AND PORATH by applying a device that allowed constant removal of the electrolysis products from the electrode vessels.

KUNKEL[73] described an apparatus that can easily be constructed in any laboratory. Two glass tubes s, 40 × 1 cm, are joined together by rubber or plastic tubing p (Fig. 8). Tube s is fitted with a glass filter f to support the starch. The connections with the electrode vessels consist of cotton wicks c enclosed in bent glass tubes or merely covered with cellophane or parafilm.

SVENSSON[74], in collaboration with FLODIN AND PORATH, designed a commercial apparatus. The glass tube is surrounded by a water jacket, and the connections with the electrode vessels and the special filling device are supplied with ground glass joints. For preparative work, columns of considerable length may be used. PORATH[69] purified posterior pituitary extracts in externally cooled columns of 300 × 1.5 cm. These experiments were carried out on cellulose powder, but starch columns of comparable or even greater length may be applied.

Preparation of starch

Homogeneous packing is essential in all chromatographic techniques where powdered materials are used. Also, in starch column electrophoresis irregular filling of the tube

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would result in tailing and spreading of the zones. The starch may conveniently be prepared as described in Part I[63]. FLODIN AND PORATH recommend the use of a starch fraction that passes a 300-mesh sieve. Application of the packing method of MOORE AND STEIN[75] also gives good results. In this technique starch is suspended in butanol and ground in a mortar. Distilled water is added to the suspension until the amount is 30 % of the dry starch. The slurry is poured into the column and slight air pressure is applied. The starch is allowed to settle during a period of one hour. Air bubbles can be avoided by dipping the empty column in water so that the filter-disc surface (Fig. 7, f) within the glass tube is covered with the liquid. The butanol above the starch is withdrawn by suction. Afterwards water is poured in very carefully and the column is connected to a Mariotte flask filled with distilled water. The water is allowed to pass through the column until an eluate shows no, or minimal absorption at 260 or 280 mμ against a water control. These washings are repeated with the buffer solution in which the electrophoresis will be carried out. The column must be kept exactly vertical during the electrophoretic run as well as during the elution.

Introduction of the sample

Before the electrophoretic run is started, the buffer solution above the starch surface is withdrawn with a pipette or driven down by slight pressure so that the upper starch layer is just dry. The sample is introduced dropwise by means of a funnel fitted with a narrow tube or simply with a pipette. The test solution is then slowly forced into the column by slight pressure. If there are components that will migrate towards the cathode, the sample must be transported over a certain distance before electrophoresis is started. In order to determine this distance the most convenient method is to add known amounts of buffer solution. The relation between the required buffer volume and the vertical displacement is easily found by trial using a coloured substance (*e.g.* haemoglobin or albumin stained with bromophenol blue). When the sample is in the desired position, the column is filled with buffer and connected to the electrodes.

Electrophoresis

As in the case of starch block electrophoresis, there are no standardized conditions for all starch column electrophoresis experiments. The most favourable voltage, current, buffer, and the duration of the electrophoretic run must be found by trial. Much depends on the packing and the length of the column. Though continuous cooling by circulating ice water allows the application of higher voltages than those used without cooling, the heat development even then represents a serious limitation of the method. In many cases asymmetric band formation causes insufficient separation. This effect becomes serious when columns with a large diameter are used. The best results are generally obtained with columns not much longer than 1 m and with a diameter not exceeding 6 cm.

CARLSON[3] observed considerable spreading of the zones when phosphate buffers

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were used. Veronal and ammonium buffers gave better results. Increasing the salt concentration had no influence upon the spreading of the protein zones.

According to FLODIN AND KUPKE[67] citrate and phosphate buffers give rise to strong electroosmotic flow in the starch medium.

In Table 3 the working conditions of a few investigators are summarized.

TABLE 3

Material	*Buffer*	*Ionic strength*	*pH*	*V*	*mA*	*h*	*Reference*
Serum lipoproteins	ammonium	0.1	9	350	—	—	3
Serum	veronal	0.05	8.6	700	25	12–20	4
Bacitracin polypeptide	acetate	0.15	4.5	—	35	44	4
Serum	veronal	0.1	8.6	400	5	18	14
Insulin activity in serum	bicarbonate	—	8.0–8.3 6.8–7.0	350	100	24	72
Thiamine and its phosphoric esters	phosphate	0.05	5.44	—	17	12	82
Vitamin B_6 compounds	acetate	0.05	5.1	—	18	14	83
Pancreatic lipase	acetate*	0.025	5.25	8 V/cm	—	48	85

* 0.005 *M* $CaCl_2$ added.

Influence of temperature

As mentioned earlier the rise of temperature as a result of the application of high voltages represents one of the most serious limitations of starch electrophoresis. In practice the relation between the applied voltage and the rise of temperature during the electrophoretic run is easily determined by measuring the temperature in the column with a thermistor at certain intervals and plotting the temperature against the time (*cf.* Fig. 3 in Part I). Only little theoretical work has been reported on the problem of temperature distribution in columns[76,77]. Recently PORATH[78] described a device for temperature measurements in cylindrical electrophoresis columns without central cooling. This author derived a fairly simple equation which allows a rough estimation of the permissible current values in an electrophoresis experiment.

The assumption is made that heat is transported only by conduction. In this case the heat flow at time t at any point of the system is described by the general equation:

$$\frac{\partial T}{\partial t} = \operatorname{div} \frac{\lambda}{c\rho} \operatorname{grad} T + \frac{Q}{c\rho} \qquad (1)$$

in which T = temperature
λ = thermal conductivity coefficient
c = specific heat
ρ = density
Q = heat generated per unit time and volume.

For cylindrical columns packed with powders like starch this equation reduces to:

$$c\rho \frac{\partial T}{\partial t} = \lambda \Delta T + Q \qquad (2)$$

in which Δ = Laplacian operator.

References p. 26.

It can be deduced that if the internally and externally cooled column were filled with buffer alone, the heat generated would be:

$$Q = 0.239 \frac{i^2}{\varkappa} \tag{3}$$

in which i = current density
$\varkappa$ = conductivity of the buffer solution
Q = heat generated (expressed in calories per cm^3).

Actually the effective cross section is reduced by the uncharged starch particles so that equation (3) becomes:

$$Q = 0.239\, k \frac{I^2}{q^2 \varkappa} \tag{4}$$

in which k = a constant characteristic for the (starch) medium
I = current
q = cross section of the column.

According to PORATH the water content in starch and cellulose differs considerably (33 and 85 % per volume, respectively). This difference is reflected in the k values.

Furthermore, PORATH evolved an equation which holds when the column is cooled with circulating water of constant temperature without central cooling:

$$T_m - T_r = \frac{Q r^2}{4 \lambda_c} \tag{5}$$

in which T_m = maximum temperature
T_r = temperature at r cm from the axis
λ_c = conductivity coefficient of the column content.

($\lambda_c = 2.2 \cdot 10^{-3}$ cal·degree $C^{-1} \cdot cm^{-1} \cdot sec^{-1}$ for starch packed in a column of 65 cm length and 8 cm diameter).

Inserting Q from equations (4) and (5) gives:

$$T_m - T_r = 0.239\, k \frac{I^2 r^2}{4\, q^2 \varkappa \lambda_c} \tag{6a}$$

$$\frac{0.239\, k}{4\, \pi^2 \lambda_c} = K$$

$$T_m - T_r = \frac{K \cdot I^2 \cdot r^2}{R^4 \varkappa} \tag{6b}$$

in which R = inner radius of the column.

Fig. 9 is the graphic representation of the latter equation, in which $K/\varkappa = 3200$, $I_I = 150$ mA, $I_{II} = 90$ mA.

For the difference between the temperature at the center T_m and the periphery T_0 (6b) simplifies to:

$$T_m - T_0 = \frac{K \cdot I^2}{R^2 \varkappa} \tag{7}$$

References p. 26.

PORATH found that k has the value 4.12 in starch columns (determined at 15° for starch–0.1 M pyridinium acetate medium).

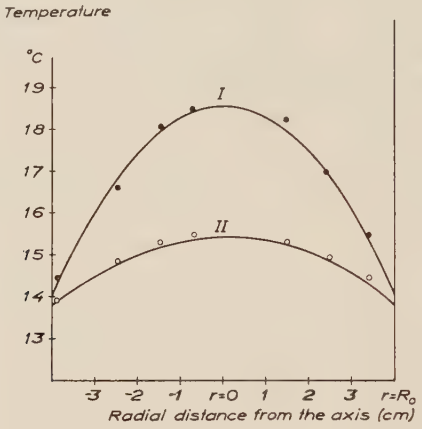

Fig. 9. Radial temperature distribution in a starch column (length 65 cm, diameter 8 cm), 12 cm below the starch surface; current in I, 150 mA; current in II, 90 mA. (Reprinted with the permission of Dr. J. PORATH.)

Elution and automatic analysis

After the electrophoretic run the electrode vessels are disconnected and removed, and a Mariotte flask filled with elution liquid is connected to the column by means of rubber or plastic tubing. The Mariotte flask is used in order to obtain a constant hydrostatic pressure during elution. It is possible to collect the separated components in reagent tubes manually, but this is a very time-consuming business, so that an automatic fraction collector becomes really indispensable. Further saving of time is obtained by analysing the eluates in a continuous analyser.

In our laboratory a very simple apparatus has been constructed, which allows

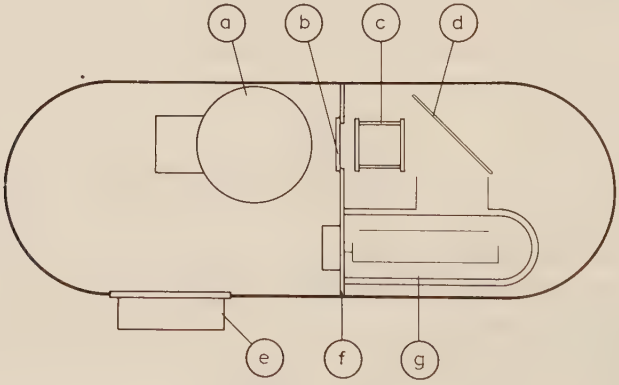

Fig. 10. Schematic diagram of the optical subunit of the fraction collector used in The Netherlands Cancer Institute, Amsterdam. (For explanation see text.)

References p. 26.

continuous estimation of nucleotides, polynucleotides, and proteins[79] (Fig. 10). The light source is a low-pressure mercury lamp, a. The lamp compartment is divided into two parts by means of the partition f, in which there is a quartz window, b. The ultraviolet light-beam falls through the glass cuvette c, which is likewise supplied with a quartz window. The cuvette has been constructed in such a way that air bubbles can pass through without adhering to the wall. The transmitted light is not measured directly but via the fluorescent screen, d. The rejected visible light is measured with a common photocell, g. If it is desired to measure at other wave lengths the mercury

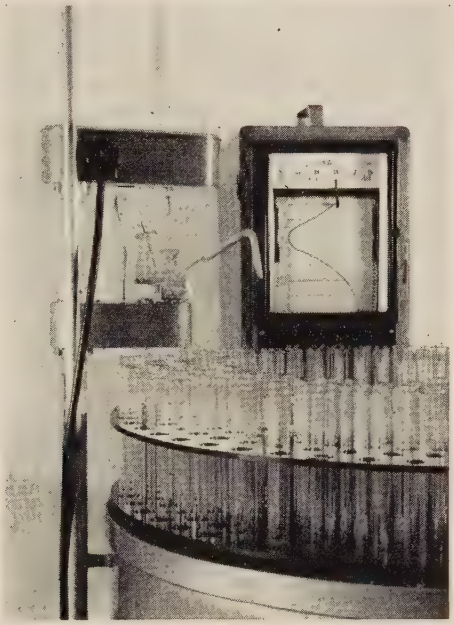

Fig. 11. Automatic fraction collector with recorder.

lamp can be replaced by other suitable light sources, if necessary in combination with filters. The optical subunit is connected to a recorder via a multipole plug, e. The fraction collector (Fig. 11) is furnished with a volume-measuring system. At the very moment that a certain tube has been filled, the connection between the vacuum tube volt meter and recorder is interrupted for a short time. The pen-recorder then falls back to its zero position. In this way, besides the registered curve, straight lines are drawn on the paper indicating the transition from one tube to the following one*.

PORATH and coworkers[87] designed an apparatus which allows continuous removal of the separated zones during electrophoresis. A buffer flow is applied in the opposite direction of the electrophoretic migration. This device permits the use of short columns and seems to counteract the broadening of zones during elution.

* Similar fraction collectors are commercially available (Dr. Gilson, Madison, Wisconsin, U.S.A.).

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Influence of electroosmosis

As described in Part I[63] the electroosmotic flow is detected with the aid of substances such as dextran or glucose that do not migrate in the electric field. However, it must be taken into account that there is some interaction between starch and glucose, so corrections must be made for the resulting retardation effect on the glucose molecules. MARCHIS-MOUREN *et al.*[85] gave examples of the electroosmotic flow under various conditions (Table 4).

TABLE 4

Buffer	*Na acetate 0.025 M + $CaCl_2$ 0.005 M pH 5.25*	*Citrate 0.025 M + $CaCl_2$ M/600 pH 6.15*	*Phosphate 0.025 M pH 6.75*	*Veronal 0.025 M + $CaCl_2$ 0.005 M pH 8.0*	*Veronal 0.05 M + $CaCl_2$ 0.005 M pH 8.0*
Potential gradient (V/cm)	6.15	9.4	8.0	6.7	6.7
Flow (cm/h)	— 0.64	— 1.1	— 0.78	— 0.51	— 0.76

According to FLODIN AND PORATH[4] monovalent buffers give rise to lower electroosmotic flow than do polyvalent buffer solutions.

3. APPLICATIONS

CARLSON[3] studied the electrophoretic behaviour of serum lipoproteins in nephrosis, diabetes, and essential hyperlipaemia.The recovery of the lipids and protein was about 100 % in monovalent buffers such as veronal. Adsorption was noticed when phosphate buffer was used. When the protein concentration was higher than 2%, spreading and overlapping of zones took place. The migration of albumin could be followed by staining with bromophenol blue prior to the electrophoretic run. The method revealed that there are at least five distinct lipoprotein components in serum.

CARLSON AND OLHAGEN[80] investigated chylomicrons in a case of essential hyperlipaemia. The chylomicron migrated as two distinct fractions. One fraction was associated with α-globulin, the other with β-globulin. These findings were in agreement with the results obtained with free electrophoresis, whereas paper electrophoresis gave only a single fraction[81].

FLODIN AND PORATH[4] separated human serum proteins and obtained patterns that agreed very well with the results obtained with other zone electrophoresis techniques. The same authors submitted an antibiotic mixture containing bacitracin to column electrophoresis on starch. Two main peaks were found. The bulk of the activity appeared to be concentrated in the largest peak. Other methods, however, revealed heterogeneity of this peak.

KUNKEL[73] fractionated serum. In some experiments the sample was made to migrate up the column; about 100 % recovery was obtained.

BOLINGER and coworkers[72] separated normal human serum. The fractions obtained were tested for insulin activity and the main activity was found in the β-globulin

References p. 26.

fraction. ^{131}I was detected in the same fraction when it was added to the serum. Furthermore, some minor activity was found in the γ-globulins, indicating that in serum endogenous insulin is linked to different proteins.

SILIPRANDI AND SILIPRANDI[82] separated thiamine and its phosphate esters. Thiamine and its monophosphate moved towards the cathode, whereas diphospothiamine and triphosphothiamine migrated towards the anode. However, cellulose powder as inert supporting medium appeared to be superior. With this medium complete separation of thiamine and its phosphoric esters could be realised and quantitative recovery of each compound was achieved.

SILIPRANDI *et al.*[83] also investigated vitamin B_6 compounds. Resolution of a mixture of pyridoxamine, pyridoxal phosphate and pyridoxine was observed, whereas pyridoxal and pyridoxamine phosphate overlapped each other partially.

MARCHIS-MOUREN *et al.*[85] achieved 135-fold purification of pancreatic lipase with a 20 % overall yield, when column electrophoresis on starch was used as final step in a fractionation scheme including ammonium sulphate and acetone precipitation as well as differential adsorption on tricalcium phosphate and aluminium hydroxide. A higher potential gradient (8 V/cm), longer duration of the electrophoretic run and the use of an acid buffer resulted in higher resolution of components than reported earlier[84].

BOUSSIER[86] obtained a pure compound of serum mucoprotein haptoglobin. Starch column electrophoresis was applied in combination with ammonium sulphate fractionation and starch gel electrophoresis.

The last-mentioned technique, which is of growing importance and may possibly supersede paper electrophoresis, will be discussed in the last part of this review.

4. CONCLUSION

Starch column electrophoresis is not used so widely as the simpler block method. A disadvantage of the former technique is the necessity of elution by means of a buffer flow after the electrophoretic run, which gives rise to broadening of the zones. According to KUNKEL AND TRAUTMAN[88] as much as a two-fold increase in the width of a haemoglobin band was found when elution over a fairly long distance through the column was required. Therefore, this procedure is not reliable in homogeneity studies, since shape and width of the bands are strongly dependent on the distance traversed during the elution. Furthermore, in some cases elution causes tailing of zones which showed no adsorption on the medium during the electrophoretic migration. From these observations it is clear that components originally separated, the mobilities of which do not differ very much, may overlap each other as a result of the transport through the column. By cutting the starch in the starch block technique, this complication does not occur.

As a rule cellulose is a better medium than starch in column electrophoresis for low molecular weight substances. FLODIN AND KUPKE[67] consider the difference in electrophoretic behaviour of these substances in paper and starch as a disadvantage

References p. 26.

of starch column electrophoresis. But this unpredictable behaviour would not really be a disadvantage if the resolving power were greater.

The principal reason why starch column electrophoresis is not so popular as the block and gel techniques is that it is not so easily manipulated as either of the two other methods and does not present appreciable advantages.

ACKNOWLEDGEMENT

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STARCH ELECTROPHORESIS

III. STARCH GEL ELECTROPHORESIS

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1. INTRODUCTION

A promising electrophoretic method was introduced by SMITHIES[5,6], who applied starch gel as supporting medium*. The idea of using gel electrophoresis was not new in itself; successful experiments with agar gels had been carried out previously by GORDON *et al.*[90,91]. The same supporting medium has already been applied as long ago as 1923 for the separation of inorganic ions[92].

Starch, as compared with other gels, has certain advantages owing to its uncommonly high resolving power. However, the reason why, for instance, serum splits up into more bands than observed in other stabilizing materials is not yet wholly clear. There is a possibility that some of the extra zones are found as a result of interaction between the borate buffer used and certain proteins linked to carbohydrate. Furthermore, a molecular sieving effect may occur. Small molecules will penetrate the swollen gel grains more easily than molecules of larger size. The same principle forms the basis of a new technique called "gel filtration" which may be successfully applied for desalting of protein solutions and separation of these solutions from peptides and amino acids[93]. In this way BOSCH AND BLOEMENDAL[94] were able to free ribonucleic acid components from sodium chloride after fractionation with salt gradients on Ecteola columns.

Whereas a growing stream of papers is being published on starch gel electrophoresis as an analytical tool, no convenient technique for large scale preparative work has so far been described. The paper by BERNFELD AND NISSELBAUM[95] deals with the separation of larger quantities of protein, but these workers used a gel that was not prepared from hydrolysed starch only.

2. METHODS

Apparatus

Starch gel electrophoresis may be carried out in similar rectangular plastic boxes as described in the first part of this review[63]. A suitable tray originally used by SMITHIES[6] had the following dimensions: depth, 6.5 mm; width, 20 mm; length, 250 mm. In

* *Note added in proof.* When this review was in print a detailed bibliography of the literature with special reference to studies of serum proteins was published by SMITHIES[151].

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starch block electrophoresis a vertical position of the box appeared to be preferable[8,63]; according to a recent paper by SMITHIES[96] a vertical arrangement of the tray also gives better results in the separation of serum components in starch gel electrophoresis. Fig. 12 shows this improved apparatus which is also used in our laboratory.

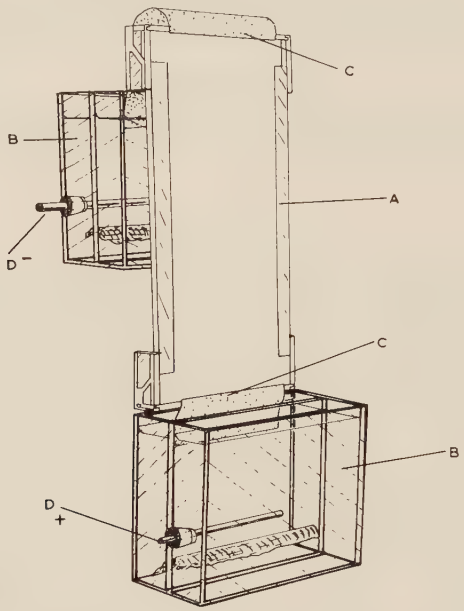

Fig. 12. Vertical starch gel electrophoresis. A. Tray containing the gel. B. Buffer vessels. C. Filter paper wicks. D. Carbon electrodes.

Electrodes

The construction of simple plastic electrode vessels is described in Part I[63]. Platinum, carbon or reversible silver–silver chloride electrodes may be used. The reversible electrodes are easily prepared by electrolysis of silver wire in 0.5 *M* HCl. The electrode is immersed in a tray containing 10 % sodium chloride solution. Filter paper or agar bridges are used to connect the electrode compartment with a vessel containing buffer solution (in serum fractionation 0.06 *M* NaOH + 0.3 *M* H_3BO_3 is mostly used).

Preparation of the gel

The essential point in the procedure of starch gel electrophoresis is the preparation of a good gel, made from soluble starch. Starch from different batches does not always possess the same gelling properties. Hydrolysed starch ready for use is now commercially available (*e.g.*, Connaught Medical Research Laboratories, University of Toronto, Canada).

Good gels are obtained in the following way:

(a) Pure potato starch is washed with distilled water and borate buffer (or any other buffer solution to be used in the electrophoretic experiment), followed by rinsing with distilled water and dehydration with acetone.

(b) 250 g of the dry starch is suspended in 495 ml acetone containing 5 ml

concentrated HCl. This mixture has to stand for 1 h at a temperature of 37°.

(c) A solution of sodium acetate or carbonate is added until the pH is 7. The starch is then resuspended in distilled water and washed thoroughly on a Buchner funnel in order to remove sodium chloride and acetate ions.

(d) The starch is dried by dehydration at 40°.

(e) The hydrolysed starch is mixed with buffer (10–15:100 w/v) with constant swirling. When a temperature of approximately 90° has been reached, the flask is de-aerated under slightly reduced pressure during a few seconds.

(f) The hot gel is poured into the tray and immediately covered with a flexible sheet of plastic, the surface of which is coated with a thin layer of paraffin oil.

(g) The excess of gel is forced out by carefully pressing on the sheet so that air bubbles are avoided. After 2–3 hours cooling in a refrigerator or in a cold room the plastic sheet is removed and the gel is covered with a perspex plate or with a layer of paraffin wax. In the latter case the melted paraffin is poured on the gel just short of congealing (temperature about 45°).

The starch may also be mixed with Hyflo-Super Cel and amylose[95], in this way a stiff gel is obtained in which the electroosmotic flow is extraordinarily low. This gel is easily cut after the electrophoretic run and preparative work becomes possible.

The gel is connected with the outer compartments of the electrode vessels by means of filter paper strips enclosed in parafilm or thin plastic sheets.

Gels may be stored overnight in a cold room or in a refrigerator.

Insertion of the sample

There are three different methods of introducing the sample.

(a) A slit is cut transversely in the gel and filled with the solution to be separated. SMITHIES' improved apparatus has a device[96] which allows the formation of a slit when the gel is poured.

(b) The sample is soaked into a small strip of filter paper. An opening is cut in the gel and the wet paper is put in by means of two forceps. When only small amounts of the sample are available, this method is preferable.

A similar insertion technique is applied when starch gel electrophoresis is carried out in combination with paper electrophoresis[97]. After the sample has been separated on filter paper, the strip is inserted into a slit in the starch gel and the second run is carried out at right angles to the first.

(c) The sample is mixed with starch powder so that a homogeneous paste is obtained. This paste is carefully put into a slit cut in the gel. When the electrophoresis is carried out vertically, melted paraffin wax is poured on the solution so that the slit filled with the sample is sealed. Then the whole gel surface is covered with paraffin wax, after which the tray is brought into a vertical position.

Electrophoresis

In serum analysis the starch gel electrophoresis is mostly carried out as described originally by SMITHIES[5,6,96] (see also Fig. 13).

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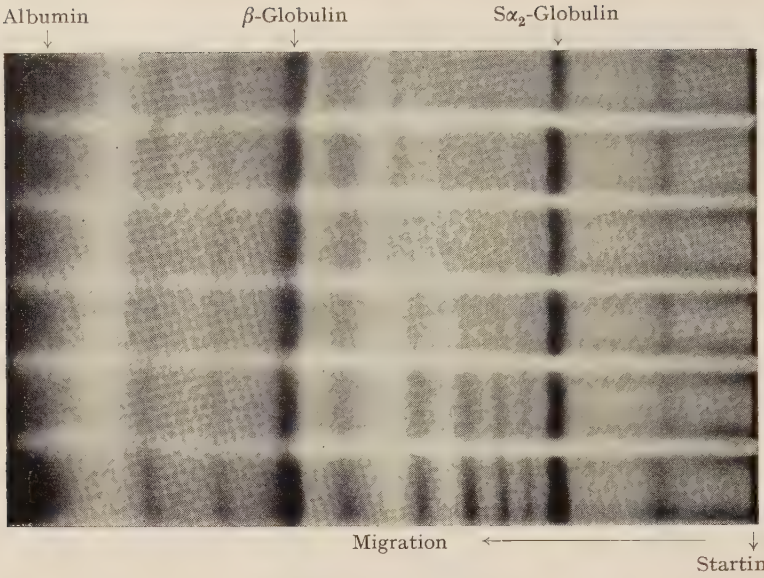

Fig. 13. Serum separation obtained with vertical starch gel electrophoresis (reprinted with the kind permission of Dr. O. SMITHIES and the Editor of *The Biochemical Journal*[96]).

POULIK[98] pointed out that a discontinuous buffer system may have considerable advantages for the separation of certain proteins.

In Table 5 the working conditions for the fractionation of several substances are given.

TABLE 5

Material	*Buffer*	*Ionic strength, molarity*	*pH*	*V/cm*	*mA*	*h*	*Reference*
Histone	Unbuffered NaCl solution (adjusted with HCl)	0.02 μ	4.1–4.9	3.2	3.5	6	101
Haemocyanin	Borate	0.02 *M* + 0.008 *M* NaOH	8.03	9	—	12	104
Haemoglobin	Borate	0.03 *M*	8.5	6	—	5	112
Human plasma	Borate	0.025 *M*	—	450 V	—	18	124
Parotid protein	Borate	0.03 *M*	8.4	8.7	4.5	5	138
Azotobacter protein	Veronal	0.1 μ	8.6	540 V	50	20	139
Gastricsin	Acetate	0.1 μ	5	4.55	—	22	144
Thyrotropin	Acetate	0.012 μ	5	260 V	11	3–4	145
Bromocresol green	Borate	0.05 *M* + 0.02 *M* NaOH	8.5	5	1.4	5.5	146

Influence of temperature

Not much is known exactly about the effect of the rise of temperature on starch gel electrophoresis. Like in other stabilizing media and in free electrophoresis the mobilities are temperature-dependent. Heat development is stronger in the strips than on the surface so that distorted zones may be the result of irregularity of the temperature

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distribution. Using low ionic strength buffers and small strips the temperature effect is reduced, provided the voltage gradient is not higher than 5–6 V/cm.According to GOLDBERG[99] the temperature at which the separation of serum haemoglobin is performed is not critical over a range of 10–25°. At 30°, however, the wicks forming the connection between gel and electrode vessel tend to dry out.

Location of substance

Different procedures are recommended for staining the gels. After the electrophoretic run the gel is carefully taken from the tray. Trays with removable side walls are very convenient. The gel is cut parallel to the bottom surface of the tray so that two equal strips are obtained. A razor blade or microtome may be used to split the gel in

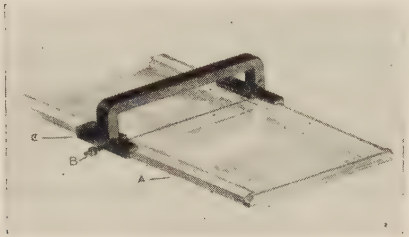

Fig. 14. Slicing device for starch gel electrophoresis. A. Bottom of electrophoresis tray. B. Screw for stretching the thread. C. Metal slide.

a single movement. In our laboratory a very simple apparatus has been constructed to divide the gels (Fig. 14). A thin stainless steel thread is stretched over a slide which fits the bottom of the electrophoretic tray.

Staining is carried out by pouring dye on the cut surface. Many workers employ protein staining with a saturated solution of amido black 10B in methanol–water–glacial acetic acid (50:50:10) during 10–30 min. Excess of dye is removed by 3–4 washings with the solvent. After the last washing the gel is kept in the methanol–water–acetic acid mixture for 1–2 h. Proteins which do not give stable complexes with amido black may be stained with bromophenol blue, light green or other dyes well-known in paper electrophoresis. Staining may also be carried out with a saturated solution of Buffalo black in a mixture of methanol–water–acetic acid (50:50:1) for 2 min. The solvent is used as washing fluid. After removal of the excess of stain the strips are left overnight in the solvent[100].

Another technique includes fixation of the gel in 5 % acetic acid before staining. In this case the colouring mixture consists of 1 g amido black 10B, 500 ml 1 *M* acetic acid and 500 ml 0.01 *M* sodium acetate. The strips are washed in 5 % acetic acid[6].

According to NEELIN AND CONNELL[101] nigrosine stains more dilute components more heavily than does amido black. However, it obscures fine zones in a densely stained region.

Lipoprotein may be stained in a solution containing 1.2 g Sudan black, 40 ml water, 600 ml ethanol and 2 ml NaOH for 16 h. Complete decoloration takes 6–8 days in 60 % alcohol[102]. A saturated solution of oil red O in a mixture of methanol–

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acetic acid-water (60:10:30) may also be used in order to make lipoprotein visible[103].

Copper-containing proteins, such as haemocyanin, are detected by placing the horizontally sectioned gel for 3–4 h in a solution containing 50 ml 10% sodium acetate and 3 ml 0.1 % solution of dithio-oximide in alcohol. A positive test results in the development of a greenish black colour[104].

Haemoglobins are detected with the benzidine reagent according to FRANKLIN AND QUASTEL[105] or as described for agar electrophoresis[106] but without zinc acetate solution. Haem proteins may also be stained by *o*-tolidine[107].

The a_2 region may be made visible by adding haemoglobin to serum protein. The result is a faintly coloured band which makes the detection of the a_2 region possible without staining[108].

OWEN *et al.*[109] use another colour reaction suitable for the detection of small amounts of haptoglobins in human serum. The complex of these proteins with haemoglobin shows peroxidase-like activity. *o*-Dianisidine appeared to be the best reagent, giving a stable brownish colour, whereas it was not absorded on peroxidase-free gel.

Quantitative evaluation of the strip

FINE AND WASZCZENKO[110] prepared gels suitable for direct photometric estimation. Since several protein dyes in alcoholic solution cause permanent opacity of the gels these authors used the aqueous staining method of URIEL AND GRABAR[111].

The strips are fixed overnight in 5 % acetic acid and then stained for 10 min. Decoloration is achieved by successive washings in 5 % acetic acid.

The stained gel is placed on a glass plate covered with 1.5 % melted agar and dried in an oven. The glass plate is withdrawn after the agar has solidified. A sheet of Whatman No. 1 filter paper is applied to the surface. The gel enclosed in agar is left in an oven at 37° for 40 h. In this way a transparent film is obtained.

A simpler method is described by VESSELINOVITCH[100]. Following staining, the strips are immersed in a 10 % solution of acetic acid and boiled for 30–60 sec. After the solution has cooled the strips may be removed and estimated photometrically.

GOLDBERG[99] applied densitometric scanning of photographs of the strips in order to obtain quantitative evaluation of the starch pattern.

The coloured bands produced by some dyes fade on storage of the gels. JACOBS[150] describes a photographic routine method which allows permanent recording of the strips.

Elution

After the electrophoretic run and staining of one of the strips cut horizontally, the uncoloured half may be cut vertically into segments of 0.5 or 1 cm width. Comparison with the stained strip makes location of the separated protein fractions possible.

The protein may be removed from the gel by freezing and thawing of the segments followed by extraction with distilled water or buffer. This procedure does not allow quantitative elution of the substance, as does the starch block technique.

Another more complicated method is digestion of the starch with amylase before protein elution.

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3. APPLICATIONS

(*a*) *Serum proteins*

Starch gel electrophoresis allows the identification of more haemoglobin fractions than do other electrophoretic techniques. ROSA *et al.*[112] observed a difference in haemoglobin pattern in six inbred strains of mice.

DE GROUCHY[113,114], who originally used the borate buffer system of SMITHIES, studied the behaviour of normal and abnormal human haemoglobin in a discontinuous buffer system (Tris–citrate–borate). Better solution was obtained and minor components, not detected by other types of electrophoresis, were observed.

GOLDBERG[99] also used a discontinuous buffer system in analyzing human serum haemoglobin.

BANGHAM AND BLUMBERG[115] studied the distribution of electrophoretically different haemoglobins between cattle breeds from Europe and Africa. There was a certain correlation between the absence of bovine B haemoglobin and a relatively high tolerance to trypanosomiasis.

Haptoglobins (= α_2-globulins) combine specifically with oxyhaemoglobin. The complex appears to be relatively stable, while uncombined oxyhaemoglobin is rapidly broken down with the formation of methaemalbumin. From these observations ALLISON AND REES[116] concluded that haptoglobins determine the renal threshold for haemoglobin. These authors ascribe haemoglobinuria to low levels or absence of haptoglobins in plasma.

Genetically controlled variations in haptoglobins of human sera were demonstrated by SMITHIES and co-workers[6,117,118].

Using borate buffer ASHTON[119] failed to detect similar differences in other mammalian sera. However, using phosphate buffer, it could be demonstrated that differences in cattle serum protein patterns were under genetic control[120]. In a preliminary communication[121] ASHTON described five serum β-globulin phenotypes of cattle. A sixth phenotype was recognized later[122] so that it was possble to propose a genetic mechanism for the system. The same author observed 3 β-gloibulin types, 5 α-globulin types, and at least 3 albumins in horse sera[123].

FINE *et al.*[124,125] found similar patterns to those of SMITHIES but the γ-globulin was distinctly divided into two components. The distribution of zones in the electrophoretic pattern was as follows: 2 γ-globulins on the cathodic side of the origin. Staining with Sudan black revealed the presence of α- and β-lipoprotein on the anodic side. Furthermore, an S-α_2 band, 9 zones between S-α_2 and the β-c-globulin, 2 F-α_2-globulins and 5 zones between the fast α_2 and the albumin were detected by the usual staining method.

HARRIS *et al.*[126] demonstrated in a series of 153 sera from Africans new β-globulins. According to SMITHIES[6,117,118] differences in these globulin patterns are controlled genetically. The author referred to the new globulins as β-CD_1 and β-CD_2. β-Globulin polymorphism in humans distinguishes the white and the black races[127]. So sera from 425 normal adult blood donors were examined by the two-dimensional method in order to demonstrate the absence of β-globulin D in whites. One-demensional starch gel electrophoresis was not always reliable for detecting this protein[128].

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ASHTON[129] obtained 8 β-globulins in sheep sera. Further separation of the zones detected resulted in 14 β-globulin phenotypes. According to that author, sheep sera show the most complex β-globulin polymorphism ever found in mammals[103].

SMITHIES AND POULIK[97] obtained originally more than fifteen resolved components from serum proteins by two-dimensional electrophoresis. In a later communication[131] the occurrence of more than twenty components was reported.

WOODS *et al.*[132] demonstrated different haemocyanines in sera of certain crustaceous species.

MOULLEC AND FINE[133] who examined the sera of 406 blood donors claim that the frequencies of the genes Hp^1 and Hp^2 do not differ very much in 40–60 % of the cases examined. This conclusion holds for most of the populations living in Europe and the white population of the U.S.A.

LATNER *et al.*[134] demonstrated that the major binding of vitamin B_{12} by serum proteins occurs by the α- and β-globulins.

FINE AND CREYSSEL[135] studied the electrophoretic behaviour of abnormal proteins in myeloma and macroglobulinaemia. A typical β-myeloma showed three abnormal components as compared with normal sera. However, macroglobulins gave no characteristic pattern.

FINE AND BURSTEIN[102] fractionated human serum lipoproteins. The most important component migrated between the origin and α-globulin. According to the authors the small mobility is caused by a high molecular weight of the lipoprotein fraction.

DE GROUCHY[136] described a method for identification of caeruloplasmin in human serum. The zone was detected by incubation with a solution of *p*-phenylenediamine. The caeruloplasmin coincided with F α_2-globulin and was absent in sera of Wilson's disease.

^{131}I-Thyroxine added to human serum was run on starch gel. The thyroxine-binding protein migrated ahead of the albumin in a sharp band in the first pre-albumin position[137].

(*b*) *Other proteins, enzymes, and hormones*

HOERMAN[138] separated parotid saliva proteins. Reproducible migration patterns were obtained in which 9–12 components were resolved. An advantage was that only small volumes of native parotid secretion were required.

PERT AND KUTT[103] observed in normal cerebrospinal fluid 10–12 protein components corresponding to the serum protein in the same individual but with different relative concentrations. In neurologic diseases considerable alterations of the protein pattern were noted.

Starch gel electrophoresis of *Azotobacter* proteins[139] revealed the presence of 12 components, differing in amino acid composition.

NEELIN AND CONNELL[101] applied starch gel electrophoresis in combination with cation exchange chromatography to chicken erythrocyte histone. Both mobility and resolution were dependent upon ionic strength, the nature of anions, pH, and on the protein concentration.

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POULIK demonstrated the complex nature of diphtheria toxins[140]. In paper electrophoresis an apparent homogeneity was observed. Furthermore the lethal factor from these toxins could be isolated [141].

DIXON AND SMITHIES[142] separated enzymes from cabbage juice. Initially the enzymes showed peptidase activity towards leucyl and glycyl peptides and glycyl transfer activity. It was more difficult to fractionate these activities by other methods.

MARKERT AND HUNTER[143] separated the esterases in B2 mouse tissues. These esterases were compared, using α-naphthyl butyrate as a substrate.

TANG *et al.*[144] studied the properties of crystalline gastricsin. When electrophoresis of the enzyme was carried out on paper and starch under similar conditions, it only migrated on starch gel. Furthermore no activity could be recovered from paper strips, probably on account of strong adsorption or denaturation. From the gel the proteolytic activity could be extracted.

PIERCE and coworkers[145] purified thyrotropin. The hormone was separated into diverse fractions after chromatography on DEAE cellulose. One of these fractions was submitted to starch gel electrophoresis and appeared to split up into six fractions These findings were in contrast to results obtained with paper and moving-boundary electrophoresis.

(c) *Non-protein substances*

Except in one instance, starch gel electrophoresis has hitherto not been utilized in the separation of organic substances other than proteins.

FRANGLEN AND GOSSELIN[146] obtained a very remarkable result. These workers succeeded in separating metastable polymers of the dye bromocresol green. From this investigation it appeared clearly that the presence of two distinct bands after the electrophoretic run in starch gel is not always an indication of heterogeneity.

4. CONCLUSION

Numerous investigations have demonstrated without doubt that—from an analytical standpoint—the starch gel technique has certain advantages over the starch column method, and even over the starch block method. For preparative purposes, however, the gel technique has up to now not been found convenient. There must be certainty that the separated fractions are homogeneously distributed through a cross section of the medium. In starch gel strips the separation is never completely homogeneous throughout the gel when the strip is sliced after the electrophoretic run. For analytical purposes this is of no importance, as only the stained surface of the strip cut horizontally is considered. In preparative work, however, the variations in different layers of the gel cause overlapping of the separated zones and the stained surface does not correspond with the pattern inside the strip.

In several cases starch block electrophoresis appears to combine the remarkable resolving power of the gel technique with the possibility of isolating the separated fraction after the electrophoretic run. So BEARN AND FRANKLIN[147] made a comparison

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of starch gel and starch block patterns of the haptoglobin–haemoglobin complexes from the three main genetic groups. Starch block electrophoresis also enabled a distinct differentiation of the three groups to be made. This was important for the isolation of sufficient material for ultracentrifugal studies.

WOODIN[148] submitted partially purified leucocidine from *Staphylococcus aureus* to electrophoresis in starch gel. Complete recovery of the protein was not obtained. On the other hand, nearly quantitative recovery was observed with the starch block technique.

In a recent paper MILLER AND BERNFELD[149] claim that a combination of starch granules and starch gel has the advantages of both media. This important finding awaits further verification.

Summarizing, it may be stated that starch gel electrophoresis is a powerful analytical tool, chiefly for the study of proteins. The utmost attention must always be paid even to the smallest detail of an obtained pattern. As a result, the success of the method depends to a great extent on the experience of the worker.

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CONTINUOUS ELECTROPHORESIS AND TWO-DIMENSIONAL ELECTROCHROMATOGRAPHY

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I. INTRODUCTION

The principle of continuous electrophoretic separation may be described as the superimposition of a direct electric current at right angles to a stream of fluid and the continuous admission of a thin stream of the mixture to be separated at a given point. Whether the stream of fluid moves freely or passes through a porous adsorbent, is of no significance. The electrically charged particles travel in the direction of the resultant, that is they are deviated more or less strongly from the direction of the stream of fluid according to their electrophoretic mobility and can, each at the end of its path, be continuously collected.

When the stream of fluid moves through a porous adsorptive system on which a direct electric field is superimposed at a right angle, and the mixture to be separated is applied discontinuously at a suitable place, the components of the mixture to be separated will move in zones, whose velocities and directions will be the vectorial sum of two velocities at right angles to each other: (a) in the direction of the electric field due to the electrochromatographic velocity and (b) in the direction of the fluid stream due to the chromatographic velocity. Hence the zones of the components are locally separated in one plane, giving a two-dimensional electrochromatographic separation.

Continuous electrophoretic separation and two-dimensional electrochromatographic separation, although they differ fundamentally in the results of their separations, are obviously subject to the same laws and can be carried out with the same apparatus, providing that a porous supporting system is used, which can act as an adsorbent for the components to be separated.

Continuous electrophoresis in an adsorptive capillary system differs fundamentally from all known one-dimensional or two-dimensional separations, regardless of whether they are chromatographic separations, separations by ion exchange, electrochromatographic separations or continuous chromatographic separations, in that a slowly moving zone can not be contaminated by a more rapidly moving one, through partial or reversible adsorption on the porous supporting medium. The continuous electrophoresis ensures that each component separated moves along its own path, thus offering a simple method for achieving absolute separations by continuous electrophoresis. This is of decisive importance for the separation of carrier-free tracers, where the smallest contamination can be the source of far-reaching error. Absolutely pure radionuclides or carrier-free radionuclides can be obtained by continuous electrophoretic separation, by using volatile or combustible background electrolytes, as the mobilities of simple inorganic ions obey individual laws in relatively large concentration ranges.

In principle, qualitative and quantitative analyses of a mixture can be carried out by this method, though it is mainly regarded as a method for preparative separations. Especially in the field of the separation of radionuclides, it should prove

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a new and very practical method of analysis. All that is necessary is to place a new set of collecting glass tubes for a known time in the apparatus, when the stationary state is reached, *i.e.* when the radionuclides leave the apparatus at the same speed as that at which they are introduced. The qualitative and quantitative radiometric analysis of the radionuclides can then be carried out as usual, providing that the introduction of the mixture to be separated into the apparatus is carried out at a known and constant speed, by means of a suitable supply pump. Another advantage of continuous electrophoresis in the separation of hazardous radionuclides is the relatively easy installation of remote control systems or even fully automatic control.

Nevertheless most of the so far published work deals with the more "classic" fields of electrophoresis: clinical biochemistry, physiological chemistry, biochemistry, pharmacy and organic chemistry.

Successful separations of serum proteins and other protein-containing mixtures, of protein hormones, toxins, antibodies, enzymes, amino acids, peptides, sugars, antibiotic substances, alkaloids, tannins, natural and artificial pigments, etc., have been reported.

At present continuous electrophoresis is still more or less confined to separations on a laboratory scale, but this limitation is only due to the lack of equipment and techniques designed for work on an industrial scale, though some research has already been carried out in this direction.

The principle of two-dimensional electrochromatographic separation was first stated by HAUGAARD AND KRONER[1] in 1948 and then in 1949 GRASSMANN[2,3] and SVENSSON AND BRATTSTEN[4] published almost simultaneously the continuous electrophoretic separation. Flat troughs, filled with powdered glass or sea sand were used as separation chambers. Then followed the publication of very much simpler experimental procedures of continuous separations on filter paper by GRASSMANN AND HANNIG[5] in 1950, DURRUM[6] and STRAIN AND SULLIVAN[7] in 1951. Since then several papers[8–13] and monographs[14–22] on electrophoresis, also dealing with continuous separations, have appeared.

2. APPARATUS

The apparatus for continuous electrophoresis must be completely dependable and meet with exacting requirements, hence although some descriptions of apparatus that can be built in the laboratory have been published[46,47] one will usually rely on commercially obtainable models, which as a rule have been developed by their manufacturers from prototypes described in the literature and further improved.

For accurate and sharp separations with the continuous method it is absolutely essential that velocity vectors of the background electrolyte, intensity of the electric field and pH at every single point of the separation area remain constant during the experiment, in spite of the Joule heat developed by the current. On the other hand it is not absolutely essential that these factors are the same at different regions of the separation area. A well-constructed apparatus, in which a filter paper suspended in a moist chamber is used as support for the separation, should fulfil all these conditions.

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This type of apparatus, because of its relatively simple construction and manipulation, is most commonly used and has already been largely employed for separations on a laboratory scale. Until recently only this type of apparatus, using moist chambers, has been commercially available.

There are some advantages in the use of trough-shaped separating chambers, filled with suitable supporting medium, in that contrary to separations on filter paper or filter paper board, the vertical speed of flow of the background electrolytes can be adapted to the requirements of the separations. Also the evaporation of the background electrolyte which can cause difficulties in electrophoresis in moist chamber, is prevented here; the temperature is kept constant through water-cooling even with high wattages; the output is considerably increased and the substances to be separated do not come into contact with the air during separation. However, such apparatus are considerably more complicated and expensive and hence have not as yet been developed to a point that would justify their general use, though their original conception is prior to that of the moist chamber type.

Some work has been done recently on free continuous electrophoresis, that is electrophoresis without a capillary supporting system. One method has been suggested in America[62] namely the continuous electrophoresis-convection method, working on the electrodecantation principle, which, unfortunately, has the drawback of only separating one pure component in one single experiment, and moreover is only applicable in the case of macromolecular substances. Another method in which a laminar stream is forced upwards through a thickened medium has also been proposed[57,58] in America. The advantage of these two methods lies in the possibility of a greatly increased output, leading to the assumption that these two methods may be the beginning of an industrial exploitation of continuous electrophoresis. In the meantime a preparative free-flow electrophoresis apparatus has been developed in Germany[60] for preparations on a laboratory scale and is marketed under the name of "Pheroplan". One of its main advantages over the filter paper curtain type of apparatus is that even strongly adsorbing substances can be continuously separated in it.

(*a*) *Filter paper curtain apparatus*

1. *Separation sheets in a moist chamber (evaporation system)*

(*i*) *Durrum type apparatus.* Fig. 1[23] shows a schematic design of the separation apparatus of DURRUM[6], which also serves as one of the simplest constructions for continuous electrophoresis. In a moist chamber (not shown in Fig. 1) a filter paper sheet is suspended over a buffer trough, in which a constant level of background electrolyte is maintained. The solution of the substances to be separated is placed over the trough in a small glass container from which it is led by a wick to a suitable spot on the filter paper curtain, under the buffer trough.

The lower edge of the filter paper curtain is serrated so as to allow each tongue to drip the background electrolyte into its collecting vessel. The two lower corners of the filter paper are left as larger strips, which dip into the electrolyte fluid, and the plati-

num electrodes are connected to a direct current source. This apparatus is very simple but it has the drawback that the migration paths are strongly curved due to the upwards decreasing intensity of the electric field. DICASTRO AND SAN MARCO[24,25] tried to overcome this disadvantage by cutting the sides of the separation sheet into three tongues which dip into a series of superposed electrode vessels. BIGHI AND TRABANELLI[26] added a pair of electrode vessels to the upper part of the original design of DURRUM. The tongues for these additional electrode vessels are at smaller distances and not placed on the sides of the filter paper sheet, but inwards to the centre, like two U-shaped cuts. The intensity of the electric field is thus strengthened in the upper part of the filter paper, where the vertical velocity of the background electrolyte is already greater, due to evaporation, and the migration paths are hence straightened.

An apparatus of the type illustrated in Fig. 1 is marketed by Shandon, London, with the addition of vertical liquid flow electrodes (Fig. 2), irrigated from two additional electrolyte containers. Their use permits great improvements in the distribution of the electric current and its transport on the filter paper, and in the continuous removal of the electrolysis products from the vicinity of the electrodes. In this application of the apparatus the electrode vessels are not used.

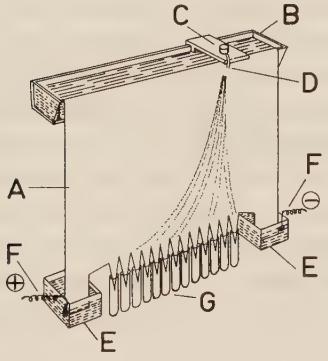

Fig. 1. Schematic representation of the separation unit of DURRUM[23]. A = filter sheet; B = buffer trough; C = substance to be separated; D = paper wick; E = electrode containers; F = platinum electrodes; G = collecting vessels.

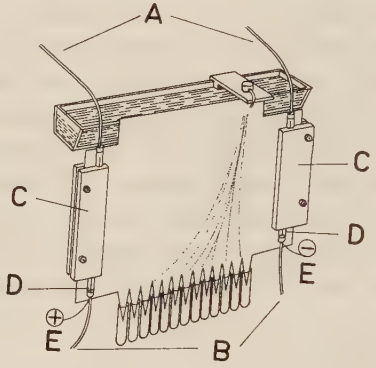

Fig. 2. Schematic representation of the vertical through-flow electrodes[23]. A = inlet of the buffer; B = outlet of the buffer; C = Perspex holder; D = cellophane tube; E = electrical connection to the platinum electrodes.

Fig. 3 shows a general view of the apparatus, produced by the Shandon Scientific Company, London. The Perspex separation unit is constructed for filter paper sheets of approximately 25 × 34 to 46 × 57 cm. The power unit supplies a filtered full-wave rectified and electronically stabilized direct current, continuously variable from 100 to 1000 V, at a maximum of 25 mA.

Fig. 4 is a schematic drawing of the separation unit of the "Spinco Model CP" made by Beckman Instruments, Fullerton, California, U.S.A.

The solution of background electrolyte is pumped electrically to the electrolyte

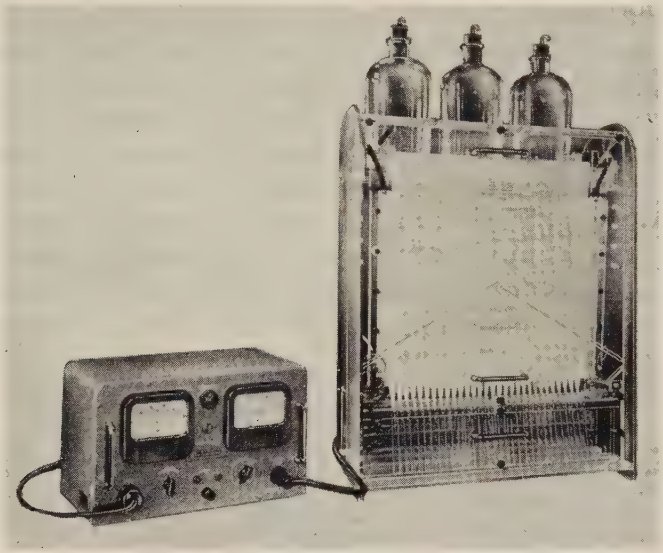

Fig. 3. General view of the Shandon (London) apparatus consisting of a separation chamber made of Perspex (on the right) and the stabilised rectifier (on the left).

vessels or the buffer trough. The separation sheet does not touch the electrolyte in the container but is supplied with the background electrolyte through a separate sheet, cut zigzag, which again is not in contact with the electric current. This arrangement avoids excessive losses of current in the buffer trough, which could result in a shunt effect.

A similar arrangement was already proposed in 1951 by BRATTSTEN AND NILSSON[28] (see also PEETERS *et al.*[37] and KARLER AND DECKARD[40]). The platinum electrodes, protected by a labyrinth system are placed in electrode vessels, which form the base

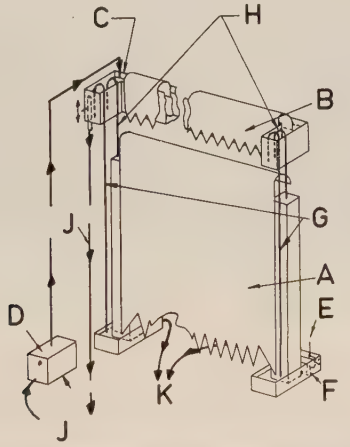

Fig. 4. Schematic drawing of the separation unit Spinco Model CP of Beckman Instruments[27]. A = separation sheet; B = curtain for the supply of electrolyte to the separation sheet; C = electrolyte container; D = electrically-operated pump; E = platinum electrode; F = labyrinth system; G = calibrated reflux capillaries; H = outlet pipes to regulate the flow of electrolyte through the wick; I = electrolyte overflow; J = electrolyte; K = mixing, rinse or discharge.

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of the apparatus. The electrolysis products are washed out of the electrode containers by calibrated reflux capillaries. A second pair of capillaries supplies the vertical wicks with electrolyte from the electrolyte container. These wicks transmit the current to the separation sheet and because they have a relatively small electrical resistance the upwards decrease of the intensity of the electric field is substantially reduced. An overflow tube ensures a constant level of the electrolyte container.

Fig. 5 gives a general view of the apparatus. The effective width of the separation sheet is 33 cm and its height 34 cm. The number of fractions can be 16 or alternatively 32. The circulation pump is built-in. The Spinco Con-Stat rectifier (not shown) permits work with a constant voltage of 100 to 1000 V or alternatively work with constant

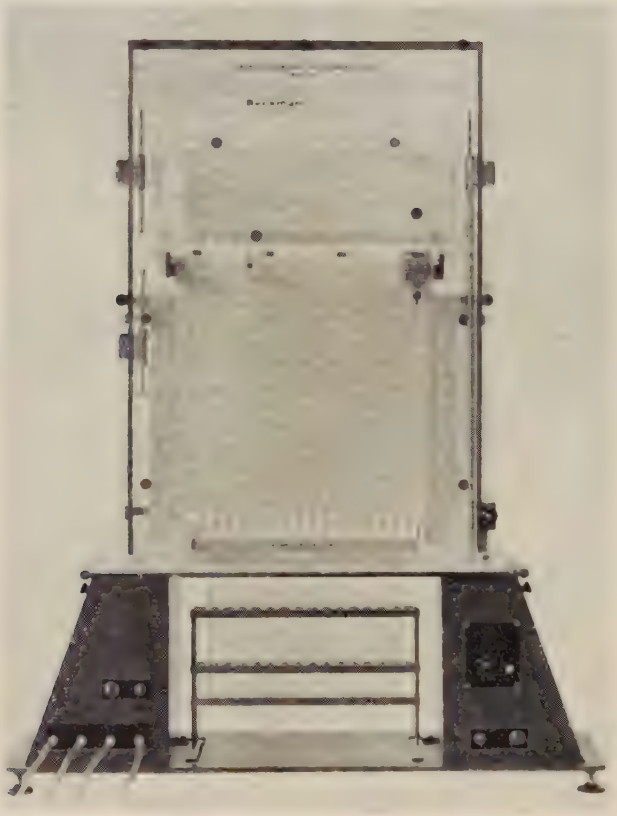

Fig. 5. General view of the separation unit Spinco Model CP (Beckman Instruments[27]). The Con-Stat apparatus for the current supply is not shown.

current between 10 and 100 mA. If requested, a specially constructed refrigerator with double glass doors is supplied, which contains the Model CP, and permits work at low temperatures (to 4°).

An automatic fraction collector is available as accessory, which can take up to six sets of collecting vessels. These can be changed at intervals of 1, 2, 3, 4, 6 or 12 hours, hence an experimental run of 72 hours is possible without supervision.

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An apparatus constructed after the classic scheme of DURRUM (see Fig. 1) is also manufactured by Jouan, Paris. Two plexiglass apparatus are supplied, the smaller with 35 and the larger with 52 collecting vessels. The stabilised voltage is regulated in intervals of 150 V from 150 V to 900 V. A similar apparatus has also been described by OSTROWSKI AND NIEWIAROWSKA-PAWLUS[30]. The dimensions of the separation sheet are: width 36 cm and height 32 cm. Both electrode containers are divided into two compartments connected with an electrolyte siphon. The lower edges of the separation sheet dip into the anterior vessels and the posterior vessels contain the platinum electrodes, continuously washed with fresh electrolyte.

An interesting microapparatus has been described by KARLER, BROWN AND KIRK[31] under the name of "micro electrochromatophoresis unit".

The apparatus (Fig. 6) is claimed to be equally utilisable for chromatography,

Fig. 6. General view of the microelectrochromatographic apparatus with built-in source of current, after KARLER *et al.*[31] (Manufacturers: Karler Laboratories Inc., Berkeley, California, U.S.A.)

analytical paper strip electrophoresis, a combination of these two techniques, for two-dimensional electrochromatography and for preparative continuous electrophoresis on a microscale.

The complete apparatus with built-in rectifier is 19.5 cm high, 17.5 cm wide and 13.5 cm deep. The separation sheet is supported by a plastic plate with pyramidal protuberances on the surface, which minimizes contact of the wet paper with the

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surface. The plate with its separation sheet can be fixed horizontally or at angles of 10° or 23°. The filter paper is cut as shown in Fig. 6. The right and left ends are folded down and dip into the electrode containers. The three upper tongues dip into the buffer trough and the lower ones, during continuous work, separate 13 fractions into the collecting vessels. The whole separation unit is enclosed in a cover of transparent plastic so as to form a moist chamber. A somewhat bigger apparatus of similar construction with 22 collecting vessels has also been described by KARLER[32,21].

(*ii*) *Apparatus based on the design of Grassmann and Hannig.* The main difference between the design of GRASSMANN AND HANNIG[20,33] and that of DURRUM is that the electrodes are placed vertically on each side of the separation sheet, in the form of platinum wires. These platinum wires, lying in special grooves are washed with an upward stream of buffer or background electrolyte from the two Mariotte bottles, which ensures the rapid removal of the electrolysis products.

Fig. 7 is a schematic design of the apparatus of GRASSMANN AND HANNIG. In earlier communications[5,34] cotton-wool pads placed on the platinum wire electrodes moistened them with buffer from the buffer trough.

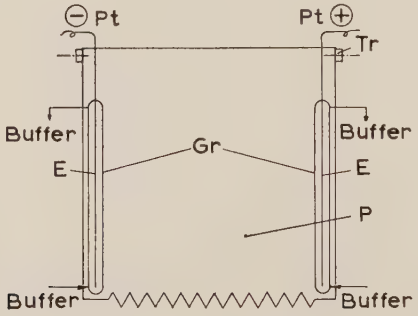

Fig. 7. Schematic representation of the design of GRASSMANN AND HANNIG. P = filter paper; Tr = buffer trough; Pt = platinum wire electrodes; Gr = electrode grooves.

Fig. 8 shows a general view of the "Elphor Va" apparatus, produced by Bender & Hobein, Munich, after the design of GRASSMANN AND HANNIG[35]. The essential part of the apparatus consists of a frame of polyvinyl chloride with built-in platinum electrodes, which holds the separation sheet. The electrodes are placed in grooves on either side of the frame, and the separation sheet is pressed by foam-rubber covered bars onto the electrode grooves. The sheet with an actual separation surface of 42 cm width and 40 cm height, terminates in 42 points, which drip into small funnels, leading outside into the collecting vessels. The separation frame is slid from above into a cemented glass case, so that it forms with the glass plate fixed on the top of the frame, a complete moist chamber. The three storage containers of the buffer are fixed to the back of the frame. They ensure a uniform flow of buffer through the electrodes and the separation sheet, by acting as Mariotte bottles. A frontal stand holds the collecting vessels for the separated substances on an adjustable platform below the glass case. A rectifier serves as current source and permits continuous adjustment of the voltage up to 800 V and a maximum current of 40 mA.

References p. 88/91.

The mixture of substances to be separated can be introduced to the separation sheet at several places through openings in the front glass wall, by means of an electrically-operated automatic supply pump. In this pump[35] the piston of an injection syringe is moved (1, 2, 3, 4 and 5 mm per hour) by means of an electric synchronous motor working with a precision gear. The speed of supply can be varied within wide limits and kept constant during the experiment by altering the cross-section of the

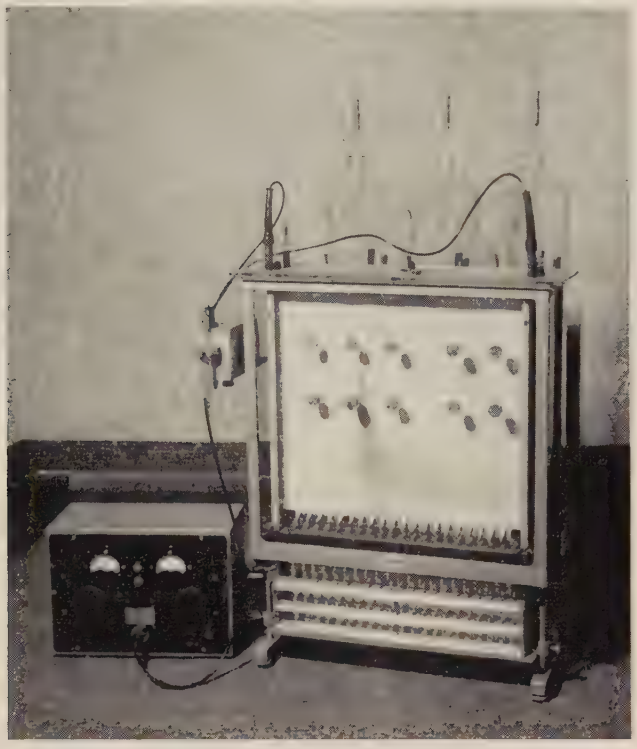

Fig. 8. General view of the separation apparatus "Elphor Va"[35] after GRASSMANN AND HANNIG. Attached to the frame on the left is the electrically-operated pump for supplying the substances to be separated (Fa. Bender and Hobein, Munich, Germany).

injection syringe and the speed of the glass piston. The volume supplied during each time-unit can be calculated from the injection syringe used and the selected piston speed.

A modification of the apparatus of GRASSMANN has been described by PUČAR[36] (Figs. 9 and 10) with special emphasis on some advantages. The filter paper can be tightened and centred at any time from the outside, without interrupting the electrophoresis, hence the paper can be introduced while still dry, then allowed to moisten itself by capillary action and finally adjusted and straightened. The dry paper can be pressed into the electrode grooves by two bars, covered with foam rubber and tightened by four knurl screws. For greater ease in manipulation the front glass wall can be entirely removed, by pulling upwards, after removal of the lid.

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The front glass wall may also be inserted after rotating it 180° around the vertical axis; then the five openings for the sample injector fall on a further four places so that without any change in the direction of the current the best position for sample injection is ready at any time. The electrode grooves have a special gas vent so that the drip of the buffer solution at the outlet is not obstructed and gases which develop at the electrodes, for example chlorine, do not come into contact with the paper or the substance to be separated. Since there is no risk of damage to the paper during its manipulation, the apparatus can be designed on a larger scale and thinner and less resistant papers may be employed. The source of current is a rectifier which regulates the voltage without graduations from 100 to 1,000 V and allows a maximal current of 50 mA.

An apparatus with cascade electrodes was recently described by PEETERS,

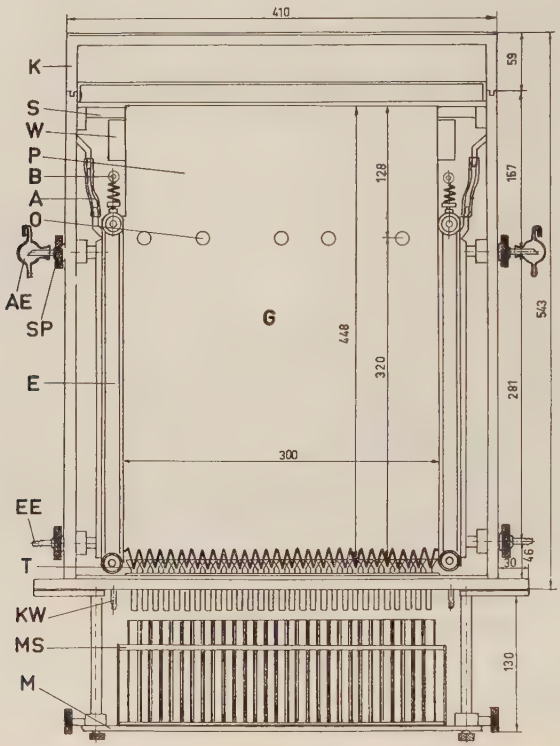

Fig. 9. Front view of PUČAR's[36] moist chamber, made of welded PVC. G = front glass wall; K = detachable top, made of PVC; S = detachable glass rod or detachable glass tube to support the filter paper; W = buffer trough made of PVC; P = filter paper; B = plug for current supply; A = gas outlet; O = openings in the glass front for the apparatus to deliver the substance to be separated; E = metal electrode supports covered with foam rubber and provided with two knurl screws for pressing the electrodes on to the filter paper; underneath the electrode supports are the electrode grooves made of PVC in which are the platinum wire electrodes; SP = device made of PVC for stretching and centering the filter paper; AE = outlet of the electrode washings with the drop-counter made of glass; EE = inlet of the electrode wash-liquids; the inlet drop counter, made of glass, is in each of the three Mariotte bottles, each of which has a capacity of 5 l. T = collecting funnels for the separated components; KW = outlet for the condensation water; M = metal tray with device for regulation of the height of the collecting vessels; MS = detachable metal stand with 24 collecting vessels, each with a capacity of 21 ml.

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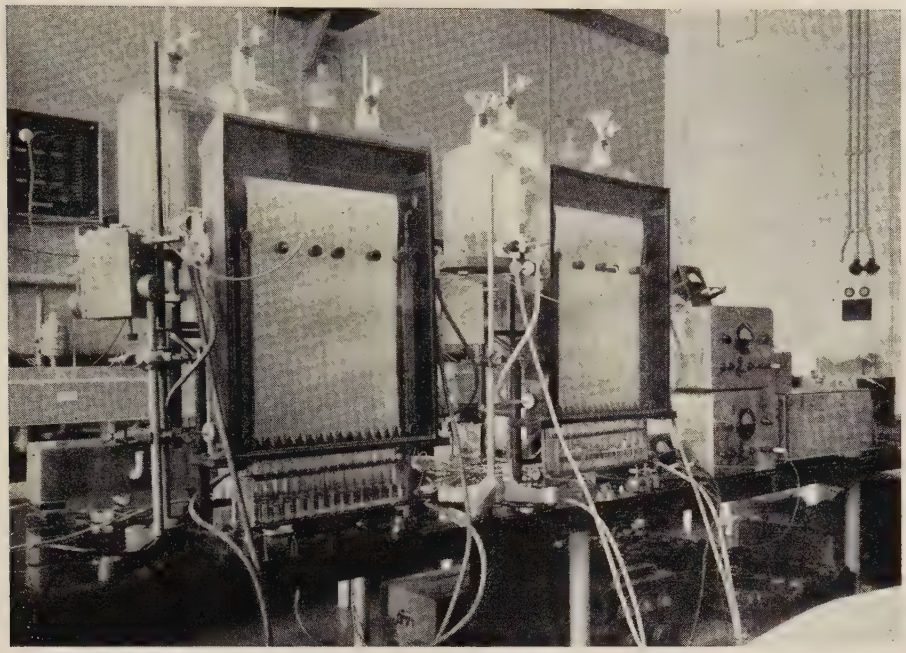

Fig. 10. General view of two apparatus in use in the laboratory of the author.

VUYLSTEKE AND NOË[37] (Fig. 11). The cascade electrodes maintain a constant hydrostatic pressure along the whole height of the vertical electrodes and thus maintain a pressure equilibrium between the electrolyte in the paper curtain and that washing the electrodes.

This is important if working without cellophane membranes (see theoretical part). The cascade electrodes are washed in a downwards direction and the paper curtain is cut zigzag also along the upper border, which is then wetted by a drip of buffer solution from the buffer trough (Fig. 12). This avoids the shunt effect of the buffer trough (see also Fig. 4 and BRATTSTEN AND NILSON[28]).

With apparatus working without cooling in a moist chamber one can count on a maximal wattage of 40–50 W. If, to improve the yield, one changes from filter paper to filter board and works with substances that show small electrophoretic mobilities, the necessary wattage increases markedly (to 250–500 W). Development of Joule heat will then considerably increase the temperature of the paper curtain and the evaporation rate. This can have serious consequences during the separation process (see Section 3(a)) and can lead to drying or even charring of the paper. As suggested by DURRUM[6], the addition of 15–20 % of glycerol or propylene glycol to the buffer prevents the evaporation and drying, but consequently increases the temperature of the separation sheet. Also it is not always easy to remove the glycerol from the collected fractions during preparative work. In general it has been observed that sandwiching of the filter board between cooled glass plates does not give the necessary sharpness of the migration tracks. This is due to the fact that the mobilities on the

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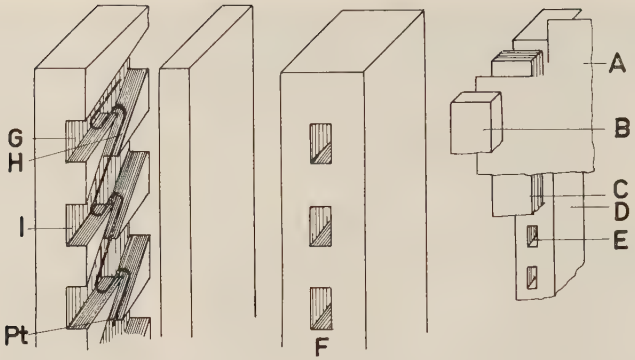

Fig. 11. Cascade electrodes (schematic), after PEETERS *et al.*[37]. On the right the attachment of the separation sheet is shown. The separation sheet A is compressed between the supports B and the electrode carrier D. Contact with the buffer takes place through the window E. Paper strips C make contact between the electrode carrier and the separation sheet. In the centre the electrode carrier F is shown. On the left is shown a vertical cross-section through the electrode carrier. The buffer solution is led into the first chamber G, comes into contact, through the window, with the separation sheet and flows, after the chamber is full, over the overflow H into the next chamber I, and so on. Pt = platinum wire electrode.

glass–paper interface differ from those inside the paper. Recently a special apparatus has been developed by HANNIG[33,38], working with high wattages on filter paper board in a moist chamber (Figs. 13 and 14). The paper board is cooled on one side by a stream of cooled air at —10° to —20°, while on the other side the liquid that evaporates is directly replaced through condensation from a second electrically heated moist board. This board is heated by being placed on a glass plate with insulated heating wires operated by a variable transformer, which selects the required temperature. A temperature difference of 5° is sufficient to effect re-condensation. The cooling temperature and degree of evaporation can be automatically controlled by the conductivity of the

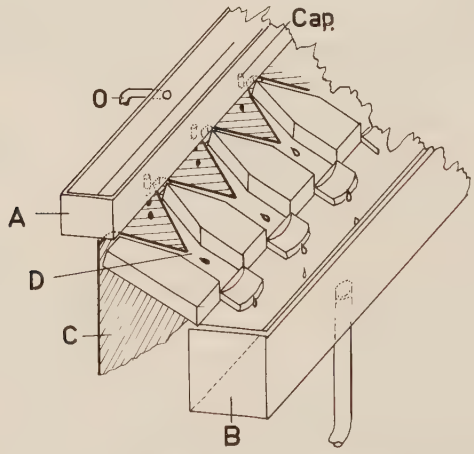

Fig. 12. The irrigation system of the separation sheet of PEETERS *et al.*[37]. The buffer solution drips out of the buffer trough A, provided with an overflow O, through capillaries (Cap.) on to the teeth cut out of the upper border of the separation sheet. Each of the teeth of the separation sheet lies in a separate channel D. The overflow fluid from all these channels is led to a collecting channel B.

separation system. At these relatively high current intensities the electrodes must be efficiently irrigated (approx. 0.5 l/min), this being done by a circulation pump. The buffer solutions from the electrode containers flow back into the storage vessel.

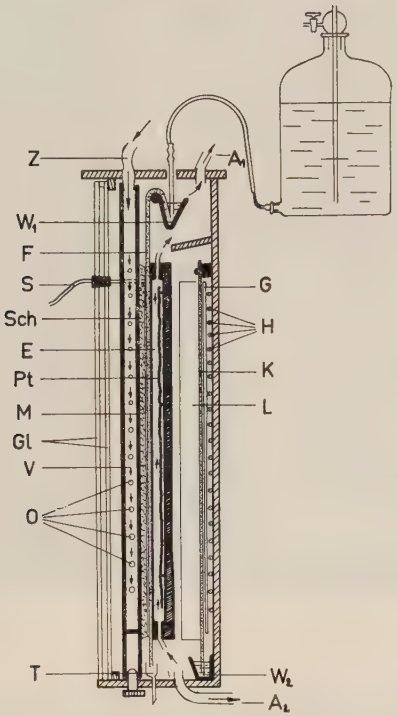

Fig. 13. A further development of the "Elphor Va" apparatus for separation on paper board with cooling with a cold stream of air from —10° to —20° and simultaneous condensation of the water, after HANNIG[38]. The separation chamber is shown schematically in lateral section. W_1 = buffer trough; F = paper board; Sch = foam rubber covering; E = electrode chamber; Pt = platinum electrodes; M = cellophane membrane; Gl = detachable glass plate (front wall); S = inlet for the substance to be separated; T = funnel; Z = inlet for the stream of cooled air; V = distributor pipe for the cold air; O = outlets for air; A_1, A_2 = outgoing air; G = glass plate, which can be heated; H = insulated heating wires; K = wettable paper board with lamellae L; W_2 = trough containing water.

Fig. 14 shows a general view of an improved apparatus with cold-air cooling and re-condensation, according to HANNIG[39]. The dimensions of the separation chamber are those of the "Elphor Va" apparatus. A stabilised rectifier is used, with a maximal voltage of 1,500 V and a maximal current intensity of 300 mA. Its separation capacity is approximately 8–10 g of protein per day.

KARLER AND DECKARD[40] described a vertical flow-electrode apparatus with a cooling tank inside the moist chamber. This tank is kept supplied with a mixture of solid CO_2 and an organic solvent. The internal air circulation of the moist chamber is ensured by a ventilator.

Fig. 15 shows a general view of an apparatus after KARLER[41] with high capacity and built-in refrigerating system.

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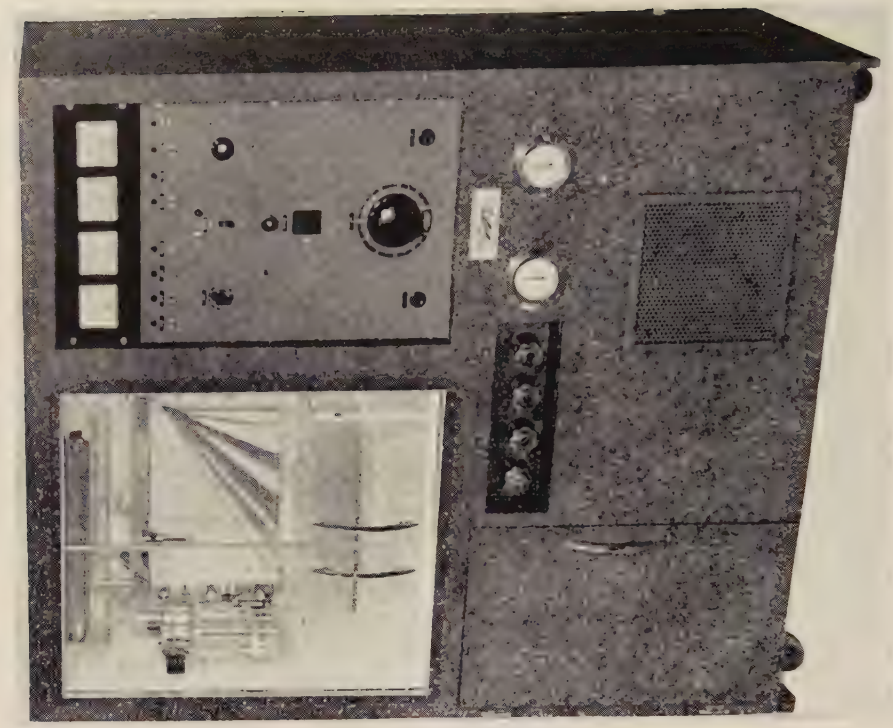

Fig. 15. General view of the new high-capacity separation unit after KARLER[41]. (Manufacturers: Karler Laboratories, Inc., Berkeley, California, U.S.A.).

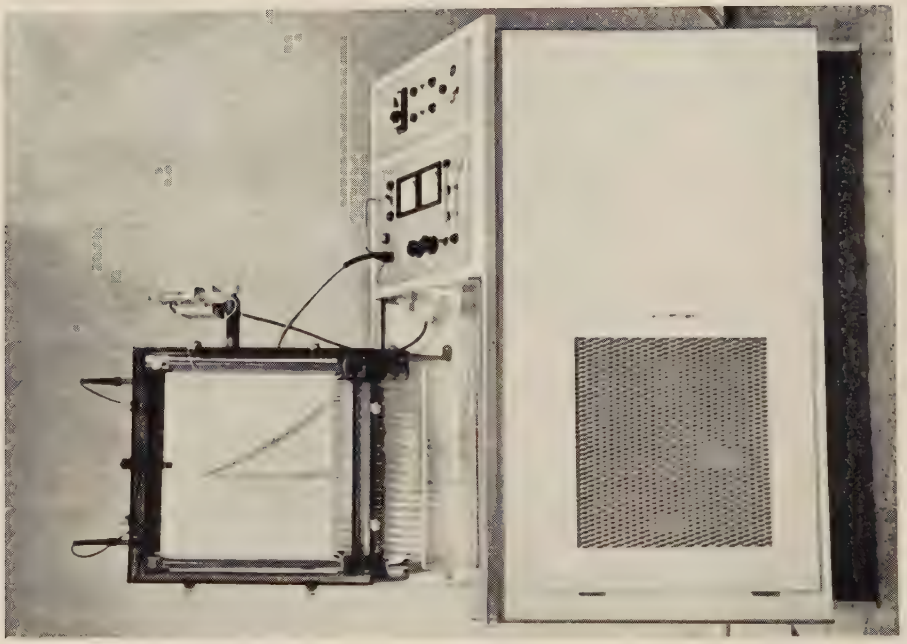

Fig. 14. General view of the separation unit "Elphor Va", after HANNIG[39] with cold air cooling. Separation capacity 8–10 g protein/day. Stabilised rectifier up to 1,500 V and 300 mA. (Manufacturers: Fa. Bender and Hobein, Munich, Germany).

(iii) Continuous electrophoresis, accelerated by centrifugal force, in a moist chamber, after McDonald. McDONALD *et al.*[42–45] use centrifugal force as driving force for the electrolyte instead of the force of gravity, the electrical current flowing perpendicularly, as usual. The horizontally rotating moist chamber (Fig. 16) consists of two

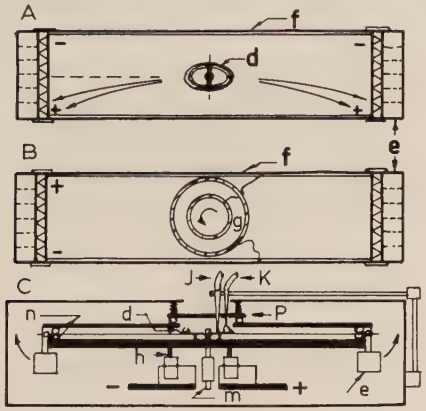

Fig. 16. Apparatus for continuous electrophoresis accelerated by centrifugal force, after McDONALD *et al.*[45]. A = view of the rotor from above; B = view of the rotor from below; C = side view of the apparatus; d = oval supply device; e = collecting vessels, which are swung up when rotating; f = platinum electrodes; g = brass collector rings; h = graphite collector brushes; j = capillary for the supply of buffer to the separation sheet; k = capillary for the supply of the substance to be separated; m = shaft of the rotor; n = silicone seals; p = non-rotating sealing unit.

shallow, rectangular dishes, the separation sheet (56 × 23 cm) being stretched horizontally between the two. The lower dish is of bakelite and the upper of lucite.

The platinum wire electrodes are placed on the two longer ends of the paper sheet. The shorter ends are cut zigzag and each point leads to a collecting vessel, of which there are nine on each side. The lower dish has two brass collector rings fixed to its lower side. The brushes lead the direct current to the platinum electrodes. The whole system is rotated in the horizontal plane by an electric motor with a speed of 400 to 900 revolutions per minute.

A special elliptical feeder of stainless steel in the vicinity of the rotation axis, permits the continuous supply of background electrolyte and the substance to be separated (Fig. 17). This feeder consists of a shallow groove forming an oval ring (greater axis 9.5 cm, shorter axis 9 cm). The outer wall of the ring slopes inwards, while the inner wall is vertical. The greater axis of the oval lies parallel to the greater axis of the moist chamber and at the end points of the greater axis a capillary opening admits the substance to be separated in a continuous stream. The feeder is fixed with two spokes to the rotation axis of the system and rotates together with the moist chamber and the paper sheet. The substances to be separated are supplied through a stationary glass capillary to the feeder and flow continuously through its two capillary openings onto the separation sheet. The buffer solution is directly supplied to the separation surface through a second and likewise stationary glass capillary also placed inside the ring of the feeder, but rather closer to the rotation centre.

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Two identical separations, mirror images of each other are always obtained. In discontinuous electrochromatographic separation the elliptical supply unit and the capillaries for the supply of the substance to be separated are omitted. The substance to be separated is then simply placed as spots on the two sides near the axis of rotation. The fact that the platinum electrodes are in direct contact with the sepa-

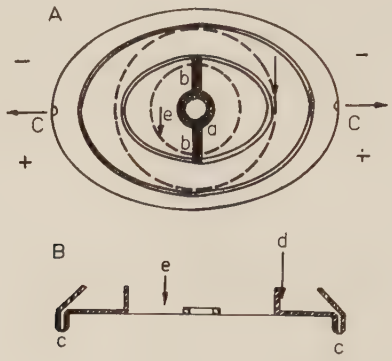

Fig. 17. Elliptical supply device for continuous supply of the substance to be separated to the separation sheet [45]. A = upper view; B = lateral view. The inner circle of broken lines indicates the buffer solution continuously emerging from the capillary e, during rotation of the rotor. The outer circle of broken lines indicates the substance to be separated emerging from the capillary d during rotation of the rotor. The substance to be separated is continuously and uniformly distributed to the separation sheet by the two capillary openings c during the rotation of the rotor.

ration sheet does not create any great difficulties in the removal of the electrolysis products.

Through the rotation the buffer is led in a circle onto the separation sheet and spreads along it in circles, hence accumulates near the electrodes and flows along them to the four outer collecting vessels, taking the electrolysis products with it. To maintain the evaporation equilibrium a very thick piece of filter paper, moistened with the buffer solution is placed on the floor of the moist chamber.

(*iv*) *Systems with separation sheets supported on a glass or plastic wall in moist chambers.* A very simple apparatus which can be assembled in the laboratory has been described by SAROFF[46]. The paper curtain is wrapped around a beaker, filled with buffer solution, with wicks leading the solution to the top of the paper curtain, from which it flows at the bottom into the collecting vessels. The platinum electrodes are placed in the electrode vessels. The electric current is also led by wicks onto the paper curtain. The moist chamber is formed by inverting a glass bell over the whole system. Although the beaker with buffer, owing to its high heat capacity, can serve as cooling agent for the paper curtain, ADAMIK[47] proposes a special method of cooling the buffer solution by the incorporation of a refrigerating coil. A system has also been described which by constant mixing of the electrode fluids keeps the pH constant.

In the apparatus of HOLDSWORTH[48] the paper curtain lies on a slightly sloped cooling surface, made of perspex, which is cooled with tap water. The moist chamber and the rest of the apparatus are also made of perspex. Vertical flow elcetrodes, similar

to those shown in Fig. 2, are used. They consist of a platinum wire in a cellophane tube irrigated with buffer solution. An automatic electric supply pump for the substance to be separated was also described. A rectifier with stabilised voltage between 70 and 840 V, which can be adjusted in intervals of 70 V and with a maximum current load of 30 mA, is used.

The apparatus of STRAIN[49] is shown in Fig. 18. In a moist chamber, constructed of polyethylene, a trapeze-shaped paper curtain lies on a slightly sloping block of polystyrene foam. The electrodes are platinum wires passed through holes along the sloped sides of the paper curtain. Sometimes free-flow electrodes made from collodion tubing, irrigated with background electrolyte, are used. The trapeze shape of the paper curtain has its justification in the fact that the intensity of the electric field

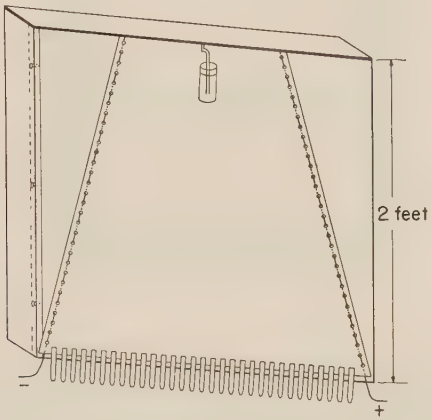

Fig. 18. STRAIN's[49] apparatus. The separation sheet is trimmed to a trapezoid shape and lies on a slightly sloping block made of polystyrene foam. The platinum wire electrodes lie along the sloping sides of the separation sheet.

and the velocity of the background electrolyte decrease downwards in the same ratio, so that the angle of the migration tracks remains constant, in a system without evaporation, and hence straight tracks are produced. However, they show a tendency to undue spreading towards their lower ends, due to influence of the horizontal flow vectors. These considerations become somewhat more complicated in systems with evaporation (moist chamber), but by taking into account the theoretical background (see Section 3(a)) a clear picture of the separation process should be obtained in each case.

2. *Paper curtains between two glass or plastic sheets (system without evaporation)*

STRAIN AND SULLIVAN[7] described a simple apparatus, which can be easily constructed in the laboratory. This "electrographic cell" is shown in Fig. 19. A specially cut paper curtain, paraffined at the places shown in the figure (B), is pressed, together with the platinum wire electrodes, between two glass plates. The paraffin is brushed onto the paper curtain as a saturated solution of paraffin in carbon tetrachloride or petrol ether and the solvent is left to evaporate. The platinum wire electrodes are placed in direct

contact with the paper curtain without any special irrigation. This causes considerable pH differences during the separation, especially around the electrodes. These electrographic cells have so far only been used for discontinuous or continuous separations of inorganic ions. Larger cells (32 cm × 32 cm) have been mounted in a steel frame, which acts as press.

In the apparatus of BRATTSTEN AND NILSSON[28] the filter paper board (40 × 20 cm)

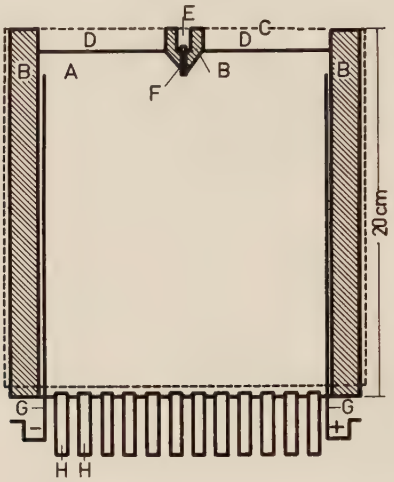

Fig. 19. The "electrographic cell" of STRAIN AND SULLIVAN[7]. A = filter paper; B = the paraffined parts of the separation sheet; C = glass plates; D = areas of the separation sheet cut out to serve as buffer troughs; E = parts of the separation sheet cut out to take up the substance to be separated; F = paper wick passing through the paraffined area; G = platinum wire electrodes in grooves on the glass plate; H = strips of filter paper to drain away the background electrolyte and the separated substance.

is cut at the upper and lower end into zigzags and is pressed between two 7 mm thick glass plates, which are coated with silicone grease to prevent adhesion between the glass and the liquid. Each glass plate has a trough of perspex on its outer side, through which tap water circulates for cooling. The upper points of the paper curtain are supplied with background electrolyte from the buffer trough through a second paper curtain, cut into corresponding zigzags, to avoid the shunt effect of the buffer trough (see also Figs. 4 and 12). The vertical platinum electrodes may be irrigated with background electrolyte if so desired. The current source supplies voltages up to 1000 V and intensities up to 250 mA.

(*b*) *Apparatus with trough shaped separation chambers, filled with porous material*

1. *Apparatus after Grassmann and Hannig*

A further development of the set-up already mentioned in the introduction[2,3,17] is reproduced schematically in Fig. 20 and a general view is shown in Fig. 21[20,51]. The separation chamber is 30 cm high, 20 cm wide and 0.8 cm deep and filled with glass

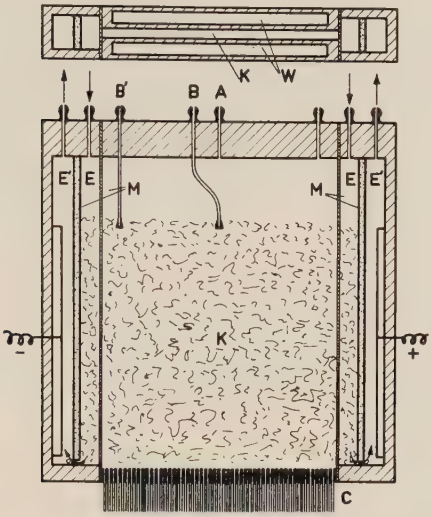

Fig. 20. Schematic representation of the separation trough of GRASSMANN AND HANNIG[51]. K = separation chamber; M = cellophane membranes or glass diaphragms; E, E′ = electrode chambers with vertical platinum electrodes; W = cooling chambers; A = supply of the solution of the background electrolyte; B, or, at choice, B′ = supply of the substance to be separated; C = 50 tubes for draining off the fluid.

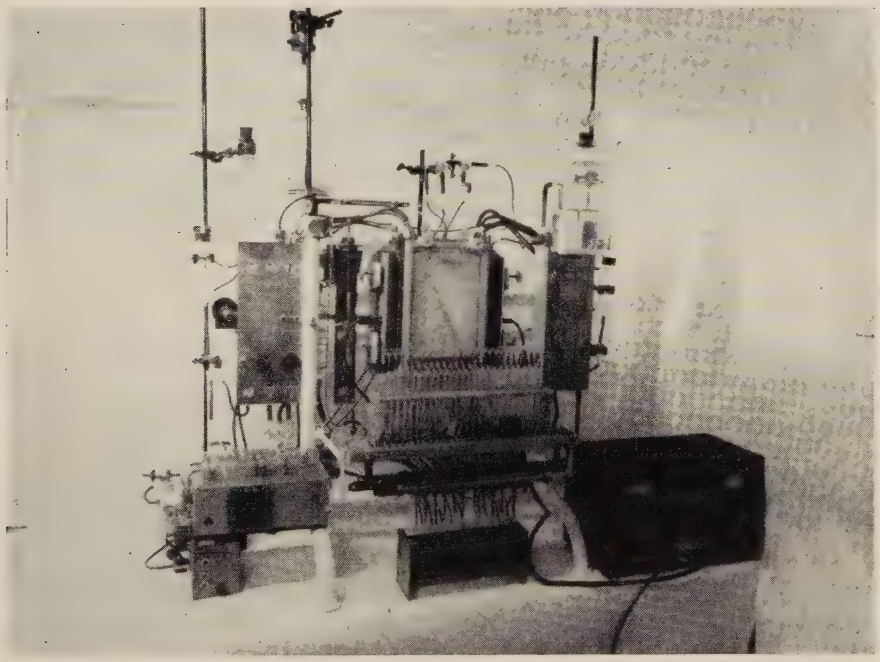

Fig. 21. General view of the separation apparatus of GRASSMANN AND HANNIG[20].

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powder, sea sand or glass beads of 0.1 to 0.5 mm diameter. The separation chamber is placed between two cooling chambers in which tap water circulates.

The separation chamber is separated from the electrode chambers by glass diaphragms or cellophane membranes. The vertical platinum electrodes are irrigated with a rapid stream of electrolyte solution. The pressure equilibrium between the two sides of the membrane or diaphragm is maintained with levelling bottles, since pressure differences could cause a lateral flow into or from the electrode chambers. The levelling bottles also serve to neutralise the electroosmotic movement in the separation chamber. 50 small tubes are built into the bottom of the separation chamber to drain off the liquid.The voltage used is 500 to 1200V and the wattage up to 150 W. The time taken by the background electrolyte to flow through the separation chamber varies between 3 and 40 minutes. Substances, whose electrophoretic mobilities differ by about 10 % hence can still be separated.

2. *Pump apparatus of Brattsten*

(*i*) *General.* At the same time as GRASSMANN AND HANNIG[2,3] were working on their first apparatus, SVENSSON AND BRATTSTEN[4] in Sweden developed independently a very similar apparatus. Essentially this consisted of a right-angled perspex trough (20 × 32 × 2 cm), carefully filled with glass powder. Its vertical electrode chambers were separated from the separation chamber itself by a perforated wall. The liquid level and hydrostatic pressure inside the separation chamber were maintained by Mariotte bottles.

Later BRATTSTEN concentrated on technical improvements of the apparatus and developed a pump apparatus[52–54]. A pumping mechanism forces the background electrolyte downwards through the glass powder in a perspex trough, which is water-cooled at the front and back. The pumping mechanism controls the speed of the background electrolyte over the whole breadth of the separation chamber. Another advantage of this apparatus is that pH or conductivity-gradients can be established for electrophoretic separations. The separation chamber itself is 28 cm wide, 30 cm

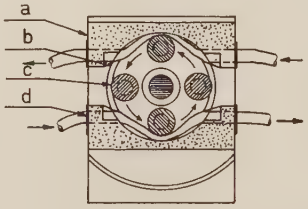

Fig. 22. Schematic representation of a peristaltic pump element after BRATTSTEN[52]. a = housing; b = rotating plate; c = rollers for compressing the tubes d made of soft rubber.

high and 1 cm deep. Along its upper edge 28 inlet nipples are provided and below 56 outlet nipples connected by polythene tubes to the pumping mechanism, which consists essentially of peristaltic tube pumps, assembled into several pumping units and operated by a common shaft. Fig. 22 shows a schematic design of a peristaltic

pumping element in action. One electric motor serves to drive all the pumping units by a chain drive. Since there are twice as many outlets as inlets, the speed of the outlet pumps is in a ratio of 1:2 to the inlet pumps. A circulation pump supplies water from the thermostatically controlled cold water bath to the cooling chambers of the trough.

The electrode chambers are fixed independently from the outside to the separation chamber and are separated from it by cellophane membranes (Fig. 23). The electrode

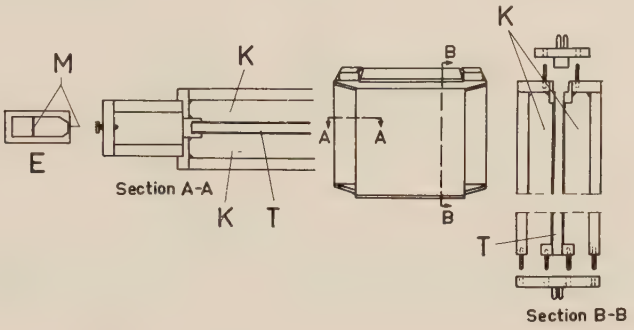

Fig. 23. The separation trough of the pump apparatus of BRATTSTEN[54]. On the left in the section A – A the electrode chamber is separately shown. T = separation chamber; K = cooling chambers; E = electrode chamber; M = membranes.

chambers are themselves divided into two by cellophane membranes. The two outer divisions, filled with liquid, carry the platinum wire electrodes and the two inner ones are filled with coarse glass powder. Two Mariotte bottles supply the electrodes with fluid, which is then pumped off to a container where it is remixed and returned by a circulation pump to the Mariotte bottles.

(ii) Automatic current and temperature control in the separation chamber. If the properties of the separation chamber remain constant the point of emergence of a fraction can be calculated from the angle of inclination α of its migration track.

$$\operatorname{tg} \alpha = \frac{v}{W} \tag{1}$$

where v = the horizontal or electrophoretic speed of the migrating fraction and W = the vertical speed of the background electrolyte, and given the following assumptions:

(1) The alteration of the vertical speed is proportional to the alteration of the speed of revolution of the motor of the pumping apparatus.

(2) Within a limited temperature range the alteration of the electrophoretic speed is proportional to the alteration of the current intensity in the separation chamber and independent of temperature variations.

(3) The exit point of a band remains constant in space and time if the revolution rate of the pumping motor and the current intensity in the separation chamber is kept constant.

(4) The apparatus which ensures a constant intensity of current controls simul-

taneously the temperature in the separation chamber. LANNEFORS[55] described a system that by keeping constant the relationship between the current in the separation chamber and the speed of the pump motor, regulates automatically the current intensity and hence also the temperature in the separation chamber.

A feed-back control system employing a transductor (magnetic amplifier) was used.

An extremely simplified drawing of the regulating system is shown in Fig. 24.

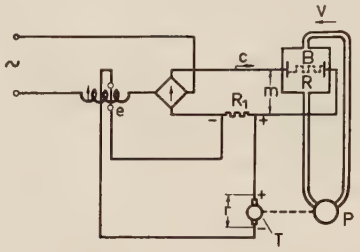

Fig. 24. Simplified schema of the feed-back control system with magnetic amplification after LANNEFORS[55]. B = separation chamber; P = pump; T = tachometer-generator whose voltage is proportional to the circulation speed of the pump.

The current c in the separation chamber is kept constant for a given speed of the pump motor (v). For example if the current intensity is slightly increased the voltage drop at the resistance R_1 also increases. The pump motor is directly coupled to a tachometer-generator. The potential drop at R_1 is coupled against the tachometer reference voltage r, giving a differential potential drop e, which, acting on the magnetic amplifier, decreases its output, and hence regulates the current intensity in the separation chamber to its original level. The accuracy of the current intensity correction increases proportionally to the magnetic amplification.

The steady-state operation of the system can be described by the following simplified equations derived from the block diagram Fig. 25.

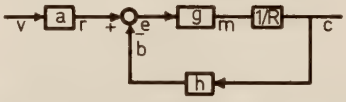

Fig. 25. Wiring diagram of the regulating system of LANNEFORS[55].

$$m = ge \qquad (2)$$

$$e = r - b \qquad (3)$$

$$b = hc \qquad (4)$$

$$r = av \qquad (5)$$

$$c = \frac{1}{R} m \qquad (6)$$

c = the controlled current in the separation chamber

v = the speed of the pump motor

r = the voltage of the tachometer-generator, proportional to the speed of the pump motor v

b = the potential drop in resistance R_1, proportional to the controlled current

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e = the actuating signal (the error) *i.e.* the difference between r and b
m = voltage applied to the bath electrodes
R = the bath resistance
g = the transductor amplification
h = the proportionality factor between b and c ($h = R_1$)
a = the proportionality factor between r and v.

Continuing equations (2)–(6)

$$c = \frac{\frac{g}{R}a}{1 + \frac{gh}{R}} \cdot v \tag{7}$$

If now the gain g is made great $\frac{gh}{R} \gg 1$

then equation (7) can be approximated as

$$c = \frac{a}{h} \cdot v \tag{8}$$

Equation (8) shows (as a and h are constants) that the relationship between the current c in the separation chamber and the speed of the pump motor v is a constant and that the current c is independent of the resistance of the separation chamber.

The pump motor is a 50 watt direct current motor, whose rotor and stator are connected in series and permit revolution speeds of 2,000 to 4,000 rev./min. It is supplied by a constant (to 1%) voltage a.c. device rectified with the aid of a selenium rectifier.

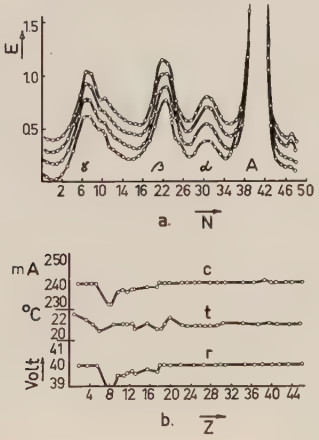

Fig. 26. Fractionation of a normal human serum, after BRATTSTEN[53]. (a) Optical density of the fractions at 284 mμ as a function of the numbers of collecting vessels. The scale of the optical density is valid only for the lowermost curve. From the other curves 0.1, 0.2 or 0.3 units must be always subtracted. N = number of the collecting vessel; E = optical density; A = albumin; α = α-globulin; β = β-globulin; γ = γ-globulin; (b) r = generator voltage; c = current intensity in the separation chamber; t = temperature of the separation chamber; Z = time.

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The tachometer-generator unit is coupled to the driving shaft of the motor.

Fig. 26 shows the 46-hour fractionation of a normal human serum as a demonstration of the postulates (3) and (4). 150 ml of the serum, containing 1.8 % of protein, were fractionated. Veronal buffer with an ionic strength of 0.03 and a pH of 8.6 was used as background electrolyte. The volume speed was 3.5 ml/h and cm^2, the current intensity 240 mA, the output 135 W, and the temperature of the separation chamber 21°. During the whole experiment, practically no fluctuations could be noted in the position of the terminal points of the individual protein fractions.

To end this section Fig. 27 shows a general view of the separation chamber and

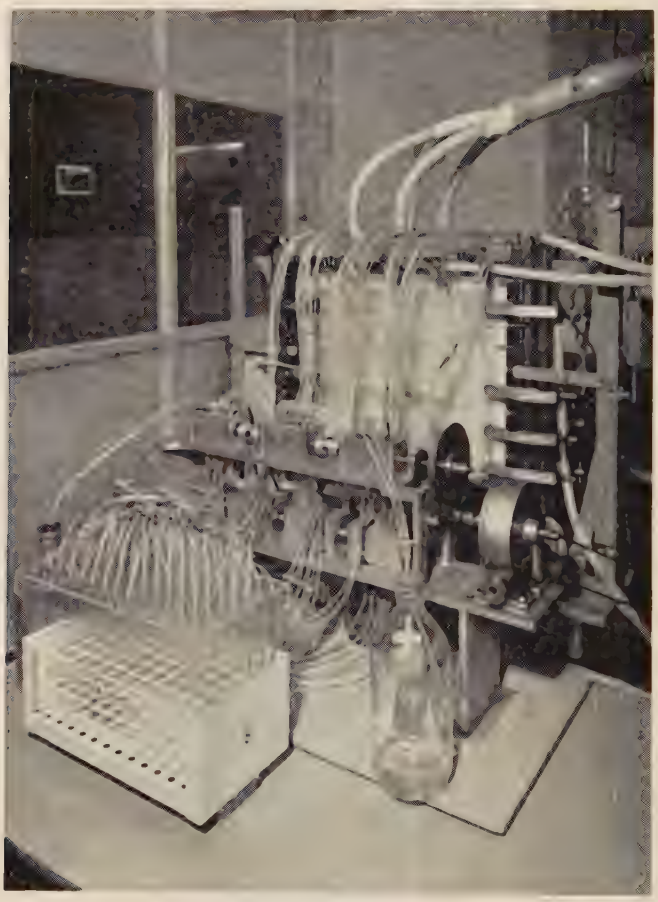

Fig. 27. General view of the separation unit and pump apparatus of the newest design by BRATTSTEN[56]. (Manufacturers: ESAB, Laxa, Sweden).

pump apparatus of the latest model, designed after BRATTSTEN[56]. It is essentially of the same type as that already described, with a similar separation chamber, regulating apparatus, cooling and pumping apparatus.

References p. 88/91.

(c) *Free continuous electrophoresis*

1. *Apparatus of Dobry and Finn with upwards directed laminar flow in a thickening medium*

DOBRY AND FINN[57,58] suggest the apparatus shown schematically in Fig. 28 whose purpose is to extend free continuous electrophoresis from the laboratory scale with an output of ml/hour to an industrial scale with an output of l/hour. The separation takes place in a perspex column of 130 × 8 × 5.5 cm. The column is of rectangular cross-section and filled with a viscous background electrolyte, which moves upwards in a laminar stream. Methocel, polyvinylalcohol or dextran are added to the back-

Fig. 28. Schematic representation of the apparatus of DOBRY AND FINN[57,58] with a laminar flow forced upwards through a thickening medium. S = separation column with the viscous fluid which is forced upwards in a laminar flow; I = slit-shaped inlet; O = slit-shaped outlets; E = electrode chambers; PW = membranes made of thermoplastic resin.

ground electrolyte to obtain the necessary viscosity (about 10 cp). The mixture to be separated is admitted continuously through a slit-shaped valve (40 × 2 mm) into the base of the column. At the top of the column the exit is also formed by several slit-shaped valves (45 × 3 mm). The electric current flows perpendicularly to the direction of the stream of viscous fluid. In the electrode chambers, the electrodes are washed from below upwards, and are separated from the column by membranes of thermoplastic resin.

The mixture to be separated flows in a single, band-shaped laminar stream from the inlet valve to the upper middle outlet valve, as long as the current is cut off; the switching on of the current causes a division into several bands, which are then collected at their outlet valves. The capacity of the apparatus is about 9 ml per min of the mixture to be separated, given a velocity of the background electrolyte of 0.5 cm per sec. So far only dye mixtures have been separated by this method. The apparatus would have to be still considerably improved for the separation of serum proteins, owing to the ten times smaller mobilities of the protein fractions. Also the conditions under which reproducible, even bands can be obtained, have to be further investi-

References p. 88/91.

gated. A whole series of factors such as the stream velocity, viscosity, buffer concentration, height of the column have so far not been thoroughly studied. Future improvements could possibly lead to large scale effective separations of enzymes and serum proteins.

2. Electrophoresis in a thin liquid layer between two parallel plates

(*i*) *Electrophoresis in density gradients, after Philpot.* In 1939, ten years before the publication of the patent of GRASSMANN[2], a continuous electrophoretic separation in thin liquid layers, had already been suggested by PHILPOT[59] having great advantages over the discontinuous method in U-tubes for the preparative separation of proteins. Fig. 29 shows the design prepared for the Discussion on the Electric Double Layer of the Faraday Society. It will only be briefly discussed here. The fluid flows in horizontal layers between two plates of perspex, the layers being stabilised through downward increasing density, from the inlet slits A (1–5) to the outlet slits B (1–5) that is

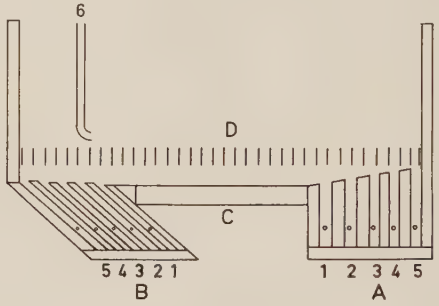

Fig. 29. Schematic reproduction of PHILPOT'S[59] apparatus. A = inlet slits (1–5); B = outlet slits (1–5); 6 = suction tube; C = carbon anode; D = copper foil cathode.

from right to left. The carbon anode C is the base and is washed by the first layer (highest density) consisting of 5 *M* ammonium bromide, giving off bromine. Layers 2, 3 and 4 are a buffer solution of 0.1 *M* ammonium chloride and 0.005 *M* ammonia. Layer No. 2 contains 4 % glycerol to increase the density, layers 3 and 4 contain 11 % ethyl alcohol. Layer 3 contains in addition 1.5 % of the protein mixture to be separated. Layer No. 5 consists of 0.5 *M* ammonium chloride, 0.5 *M* hydrochloric acid and 40 % methyl alcohol.

The cathode D is composed of a vertical row of copper foils. The protein mixture is separated into fractions in the vertical electric field and leaves the apparatus through the outlet slits (2–4) where they are scanned by a Schlieren system. The normal current intensity is 6 A and should be increased to 100 A if the apparatus is enlarged in two dimensions.

(*ii*) *Apparatus without supporting medium, after Barrollier, Watzke and Gibian.* BARROLLIER, WATZKE AND GIBIAN[60] recently described an apparatus that operates without supporting medium or even without a thickening agent (Fig. 30).

The buffer solution flows slowly as a 0.5 mm layer between two cooled, almost

horizontal glass plates. The glass plates are about 60 × 60 cm. The buffer solution is introduced from behind by a cellulose strip, which is wetted by seven stainless steel cannulae fed from a Mariotte bottle. The electric current flows transversely to the direction of the buffer flow. Contact is made by strips of filter paper which dip into

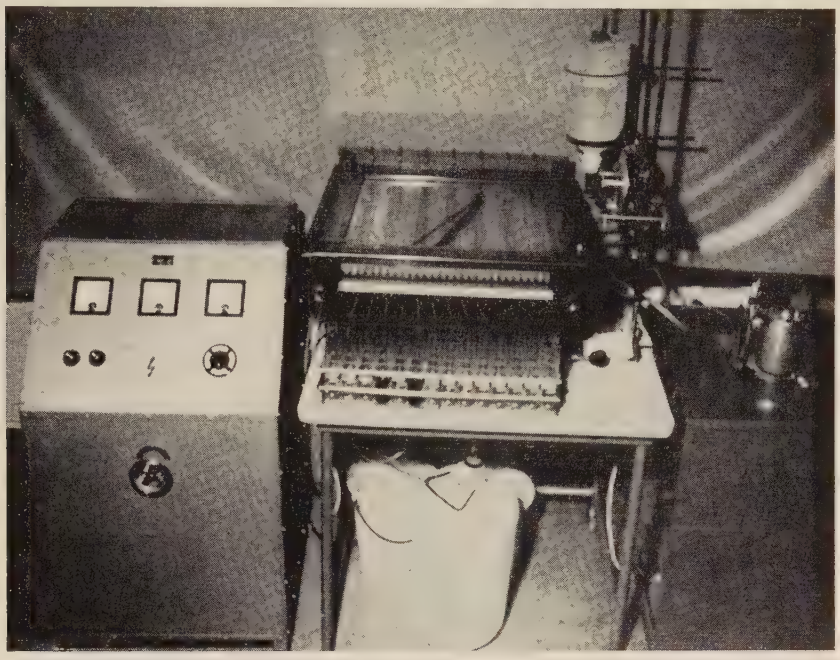

Fig. 30. General view of the free-flow apparatus "Pheroplan"[61] without a supporting medium, after BARROLLIER, WATZKE AND GIBIAN[60].

lateral electrode troughs. At the front of the glass plates 56 narrow cellulose strips inserted between them hold them apart at the desired distance and at the same time remove the buffer, which drips off their points.

The electrode troughs are washed with buffer from a 25 l plastic bottle, by means of a diaphragm pump. The substances to be separated are introduced through a hole drilled in the upper plate through a glass capillary supplied by a peristaltic tube pump. An adjustable transmission controls the pump output, which can be continuously regulated from 0.5 to 8.8 ml/hour. The buffer layer takes between 45 to 60 minutes to traverse the apparatus. The refrigerator, which has a circulation thermostat to maintain the temperature of the circulating liquid at a constant level, has an output of about 1,500 kcal/h. The pump output is 6 l/min. The source of the current is an electromagnetic constant voltage device which can be continuously regulated from 500 to 3000 V. The maximum current load is 500 mA.

3. *Continuous electrophoresis-convection apparatus after Bier*

BIER[62] described an apparatus which, like the one already described, works without

thickening medium and with the relatively large separation capacity of 4 ml/min. This apparatus is based on the principle of electrodecantation of PAULI[63] and hence is only suitable for separations of high molecular ampholytes. Only one component of the mixture can be isolated in each separation. The apparatus (Fig. 31) consists essentially of two outer semi-permeable membranes, set parallel to each other at a distance of 3–4 mm and a third semi-permeable membrane, placed between them but not reaching to the base of the unit.

The cell is thus divided into three compartments, two at the top and a common one at the bottom. All three compartments have means for continuous input or withdrawal of the colloid solution. The cell is immersed in a circulating and cooled buffer solution and a direct current electric field can be established across the membranes. The current intensity is 2 A and membranes of sizes ranging from 13 to 125 cm^2

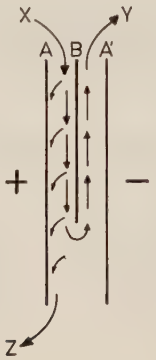

Fig. 31. The electrophoresis-convection apparatus of BIER[62] (schematic). A, A′ = outer membranes; B = intermediate membrane; X = inlet for the solution; Y, Z = outlets for the solutions.

were used. If the current intensity is kept constant, any changes in the membrane surface area have little influence on the separation effect.

For the separation of a protein mixture the pH of the buffer is adjusted close to the isoelectric point of one of the proteins and so that the others all have charges with the same sign. The polarity of the current is so selected that the mobile protein migrates toward the outer membrane of the compartment and forms a layer of increased concentration and density in the immediate vicinity of the outer membrane and will be withdrawn quantitatively in that part of the liquid which is collected through the bottom compartment. The other protein, at or close to the isoelectric point, will not be influenced by the electric field and hence flows with the liquid to Y, where it is withdrawn. Only this component can be obtained in the pure form, because the mobile protein fraction collected from the bottom compartment is still contaminated with the isoelectric protein in its original concentration.

This principle was already applied for discontinuous separations in 1920 by SAMEC[64–66], in 1941 by KIRKWOOD[67] and in 1943 by GUTFREUND[68]. KIRKWOOD *et al.*[69,70], published in 1950 a quantitative theory of convection electrophoresis.

References p. 88/91.

3. GENERAL

(a) *The process in a moist chamber*

In continuous electrophoresis the movement of an electrically charged particle, reversibly adsorbed onto a supporting medium (separation sheet etc.) can be expressed according to PUČAR[36] as the vectorial sum of the horizontal or electrochromatographic and the vertical or chromatographic components. For greater simplicity we will first consider the horizontal component for an unadsorbable particle under conditions for continuous electrophoresis in a moist chamber, which can then be considered as a purely electrophoretic component.

1. *The horizontal or electrophoretic component*[36]

In electrophoresis in a moist chamber the intensity of the electric field is not constant in the horizontal direction due to evaporation of the solvent, inducing a concentration of the electrolyte, and due to the horizontal capillary flow in the filter paper[71,72].

In the stationary state the electrolyte solution flows vertically from the buffer trough (W), as well as horizontally from the electrode grooves (E) onto the filter paper. Fig. 32 shows a schematic design of the distribution of the electric field intensity (H-curves) on the filter paper in the stationary state and in four typical horizontal planes.

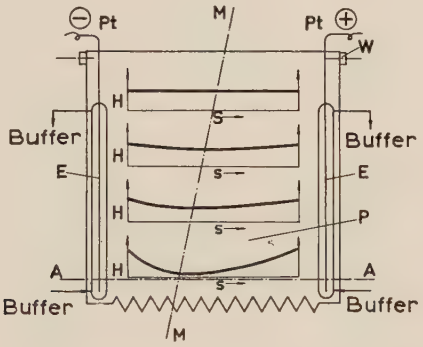

Fig. 32. Schematic representation of the intensity of the electric field (H-curves) in the stationary state on the filter paper[36]. The curvature of the H-curves increases from above downwards in the stationary state because of evaporation and capillary action. P = filter paper; W = buffer trough; E = electrode grooves with platinum electrodes; M—M = position of the minima of the H-curves, or the maximal electrolyte concentration in the horizontal concerned; A—A = a characteristic horizontal.

We will now consider an anodic electrophoretic movement of the electrically charged particles. A cathodic movement would resemble the anodic movement in principle. We shall leave aside the fact that the mobility of the particle (u) cannot be constant due to migration through concentration gradients of the electrolyte and the resulting alteration of the ζ potential.

The apparent velocity of a particle of mobility u in an electric field H is[71]:

$$v' = uH - w' \qquad (9)$$

Here w' represents the sum of the horizontal capillary flow and the electroosmotic flow. Differentiation with respect to s gives:

$$\frac{dv'}{ds} = u\frac{dH}{ds} - \frac{dw'}{ds} \tag{10}$$

From this follows

$$\text{(a)} \quad u\frac{dH}{ds} = \frac{dw'}{ds}, \qquad \frac{dv'}{ds} = 0, \qquad v' = \text{constant} \tag{11}$$

The particle moves in a horizontal direction at a constant speed

$$\text{(b)} \quad u\frac{dH}{ds} > \frac{dw'}{ds}, \qquad \frac{dv'}{ds} > 0 \tag{12}$$

The particle undergoes an apparent acceleration of movement in the horizontal direction

$$\text{(c)} \quad u\frac{dH}{ds} < \frac{dw'}{ds}, \qquad \frac{dv'}{ds} < 0 \tag{13}$$

The particle shows an apparent horizontal retardation.

Equation(10) can be demonstrated simply as shown in Fig. 33. The upper diagram in Fig. 33 shows in the plane A–A (Fig. 32) the following curves: (1) the curve of the

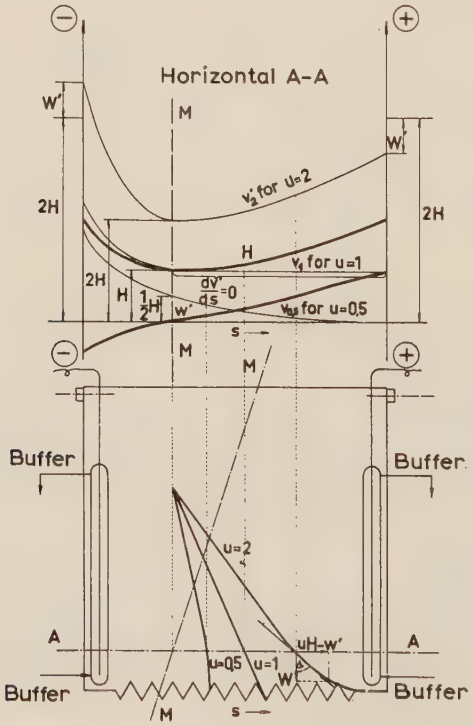

Fig. 33. Above: the H- and w'-curve in the horizontal A—A in the stationary state (schematic)[36]. According to equation (9) the curves are shown of the apparent horizontal speeds v'_1 for $u = 1$, v'_2 for $u = 2$, and $v'_{0.5}$ for $u = 0.5$ in the horizontal A—A. Below: schematic representation of the paths of the particles on the filter paper with the mobilities $u = 0.5$, $u = 1$, and $u = 2$.

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electric field strength H, and (2) the curve of the sum of the horizontal capillary flow and of the electroosmotic flow w'. Further according to equation (9) the apparent speed curves are shown, v'_1 for the mobility of the particles $u = 1$, v'_2 for $u = 2$, and $v'_{0.5}$ for $u = 0.5$. To simplify the gradients dH/ds and dw'/ds are shown of equal size at the right of the M–M line in Fig. 33. The M–M line is the position of the minima of the H-curves.

The particle with $u = 2$ always experiences an apparent acceleration in the horizontal direction, on the right of the M–M line; in the same region the particle with $u = 1$ moves at a constant speed and the particle with $u = 0.5$ undergoes a retardation of its movement. Since it was assumed that none of the three particles is adsorbed on the filter paper, the vertical component is, for all three particles, equal to the vertical electrolyte speed W.

The lower diagram in Fig. 33 shows schematically the tracks of the particles on the filter paper. To the left of the line M–M, all three particles experience apparent horizontal retardation and if the starting point was shifted still more to the left, the tracks of the particles on the filter paper would be S-shaped. Though a small portion of the tracks in Fig. 33 are to the left of the line M–M, this retardation at the beginning of the tracks was neglected, as the curves and the effects of the curves H and w_1 on the upper part of the paper (at the beginning of the development) are minimal. The angle of the M–M line is the result of electroosmosis.

Recently PEETERS, VUYLSTEKE AND NOË[37] found that during electrophoresis in a moist chamber a characteristic distribution of the background electrolyte ions appears as soon as the filter paper has reached equilibrium.

2. *The vertical or chromatographic component*

STRAIN AND SULLIVAN[7] already mentioned that the continuous separation of two substances is not possible if the relative ratio of their electromigration and chromatographic migration is the same. In the discontinuous process the separation of two substances only becomes impossible if their electromigration and chromatographic migration are identical. See Fig. 34.

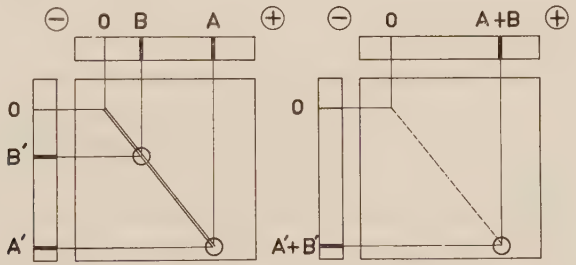

Fig. 34. The separation of substances A and B[36]. Left: the substances are separable by the discontinuous process. The separability depends on the chromatographic effect only. The electrophoretic mobilities are identical and the two substances are therefore inseparable by the continuous process. O = starting line. Right: the chromatographic and the electrophoretic effects are identical and therefore the two substances are also inseparable by the discontinuous process.

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In both cases the electrophoretic mobilities (u) of the substances A and B are identical. The substances A and B in the left hand figure are actually effectively separated with one-dimensional strip electrophoresis, but the separation itself is obviously due to a chromatographic effect. There is no electrophoretic separation. In the right hand figure there is neither an electrophoretic nor a chromatographic separation effect and hence a discontinuous (electrochromatographic) separation as well as a continuous (electrophoretic) separation is impossible.

Fig. 35 shows a continuous or discontinuous separation. The substance B is adsorbed on the filter paper, the substance A, however, is not adsorbed but moves vertically with the liquid front, that is with velocity W. For greater simplicity the

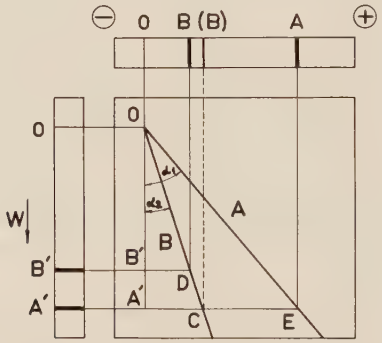

Fig. 35. The separation of substances A and B, which show different chromatographic and also electrophoretic characteristics[36]. The separation is possible by both continuous and discontinuous process. Further explanation in the text.

tracks $\overline{OA}$, $\overline{OB}$, $\overline{OA'}$ and $\overline{OB'}$ are assumed to take place in a system without evaporation. The distance $\overline{OB}$ is then the apparent electrophoretic path of the substance B; the remaining ones are the distances of the true electrophoretic or chromatographic paths. During the time in which the substance A travels the distance $\overline{OE}$, the substance B only moves from O to D, due to its adsorption on the filter paper. If this was not the case, it would move in the same time to the point C. If we project the distance $\overline{OC}$ onto the horizontal line we obtain the distance $\overline{O(B)}$ that the substance B would have travelled in paper strip electrophoresis, if not retarded through adsorption on the filter paper.

When, during the continuous process, the two substances have arrived at the bottom of the filter paper, then the path of the substance B is saturated in the stationary state and any further substance is no longer adsorbed. This, however, does not alter the path of substance B, as the projection of $\overline{OC}$ to the horizontal as well as to the vertical line corresponds to the paths without adsorption.

The electrophoretic mobility can be calculated from a measurement of the apparent path that a substance travels on an adsorbent. For a system without evaporation the following holds

$$u = \frac{\overline{O(B)}}{H\tau} \tag{14}$$

$$\overline{O(B)} = \frac{\overline{OA'}}{\overline{OB'}}\overline{OB} \tag{15}$$

and for

$$\frac{\overline{OB'}}{\overline{OA'}} = R_F \qquad u = \frac{1}{R_F}\frac{\overline{OB}}{H\tau} \tag{16}$$

or

$$u = \frac{u'}{R_F} \tag{17}$$

Here u represents the true mobility, u' the apparent mobility on an adsorbent, τ the time and R_F is the well-known chromatographic factor. According to (17) the horizontal component is:

$$v' = R_F v \tag{18}$$

or

$$v' = R_F u H \tag{19}$$

and the vertical component is:

$$W' = R_F W \tag{20}$$

and

$$\operatorname{tg}\alpha = \frac{v'}{W'} = \frac{uH}{W}$$

finally:

$$\frac{\operatorname{tg}\alpha_1}{\operatorname{tg}\alpha_2} = \frac{u_1}{u_2} \tag{21}$$

For the process in a moist chamber:

$$\operatorname{tg}\alpha = \frac{uH - w'}{W}$$

and for the same point ($w'_1 = w'_2$):

$$\operatorname{tg}\alpha_1 - \operatorname{tg}\alpha_2 = \frac{H}{W}(u_1 - u_2) \tag{22}$$

The angles of the paths of the particles, in a system without evaporation, are proportional to their electrophoretic mobilities (21). In electrophoresis in a moist chamber the differences in the angles are proportional to the differences between their mobilities in reference to a given point ($w'_1 = w'_2$) (22). For a system without evaporation the same holds, but now it applies to all the points on the filter paper, because $w' = 0$. Hence in the continuous process two substances are only then separable if they have different electrophoretic mobilities. If adsorption occurs it has no influence on the separation. In other words: all particles which are on the same path have identical electrophoretic mobilities.

Every discontinuous process is essentially an electrochromatographic process. The only substances which cannot be separated are those which have at the same time identical electrophoretic mobilities and identical R_F values. In one-dimensional strip-electrophoresis or one-dimensional electrochromatography those substances cannot be separated to which equation (23) applies:

$$u_1 R_{F_1} = u_2 R_{F_2} \tag{23}$$

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Table 1 gives a summary of electrophoretic and electrochromatographic processes and also of the conditions under which no separation takes place.

TABLE 1

SUMMARY OF ELECTROPHORETIC AND ELECTROCHROMATOGRAPHIC PROCESSES, AFTER PUČAR[36]

Process	*Conditions for inseparability*
Continuous electrophoresis	$u_1 = u_2$
Two-dimensional electrochromatography or two-dimensional electrochromatophoresis (discontinuous electrophoresis) (The condition for this process is simultaneous separation in the chromatographic and the electrophoretic direction)	$u_1 = u_2$ and, at the same time, $R_{F_1} = R_{F_2}$
One-dimensional electrochromatography or one-dimensional electrochromatophoresis	$u_1 = u_2$ and, at the same time $R_{F_1} = R_{F_2}$ or $u_1 R_{F_1} = u_2 R_{F_2}$
(One-dimensional) strip electrophoresis (R_F values for all fractions are 1)	$u_1 = u_2$

Fig. 36 is an illustration of the relationships just discussed. A dyestuff mixture was separated, on the left, by continuous electrophoresis, and on the right by discontinuous or two-dimensional electrochromatography under the same conditions. The dyestuff mixture was composed of: Tropeolin O (T), water-soluble fluorescein (F), chlorophenol red (P), rosolic acid (R), chrysoidine Y (Ch) and nitraniline (N). The concentration of each of the six dyes in 50 % ethanol was 4 mg/ml. The electrolyte used was a boric acid–sodium acetate–NaOH buffer solution with a pH of 8.6 and an ionic strength of $\mu = 0.012$. The filter paper was Munktell No. 20/150; the time between the entry and exit of the substance was 3.5 h (the vertical buffer speed); the voltage was 400 V and the current strength 20 mA. The size of the filter paper can be inferred from Fig. 9. In Fig. 36, on the left, a chromatogram is shown and below a one-dimensional electrochromatography of the same mixture of dyes. From Fig. 36 it is evident that, among other things, fluorescein (F) has a higher electrophoretic mobility than chlorophenol red (P), whilst the electrochromatographic mobility of fluorescein (F) is lower than that of chlorophenol red (P). Tropeolin O (T) is differentiated from chlorophenol red (P) much more in respect of its electrophoretic mobility than it is in respect of its electrochromatographic mobility. Rosolic acid (R) is electrokinetically separated into two fractions (R_1 and R_2). The electrophoretic mobility of both these fractions is significantly greater than their electrochromatographic mobilities.

STRAIN[12,73] doubts that the chromatographic effect is the same in the horizontal as in the vertical direction. The chromatographic retardation in the direction of the electric current should differ from the chromatographic retardation in the direction at right angles to the electric current. The fact that Ag and Ni ions, for

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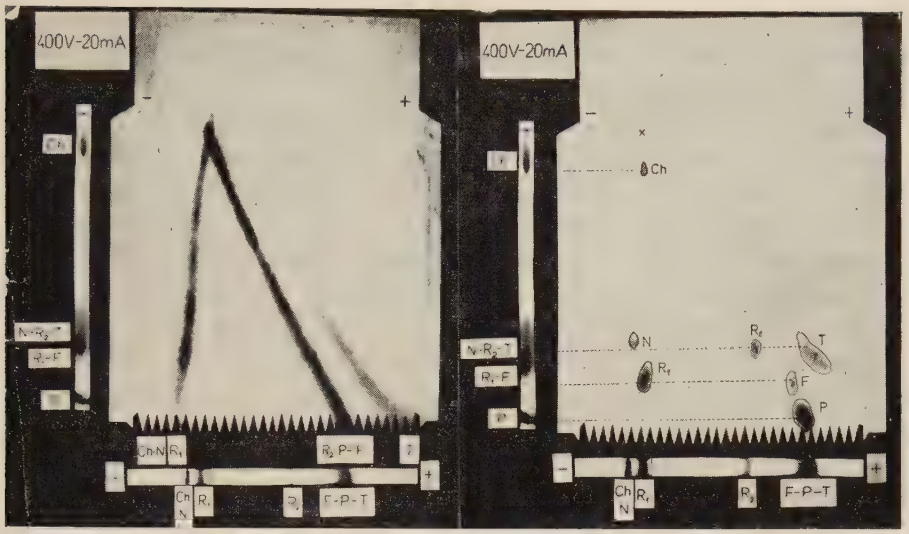

Fig. 36. Comparison of the continuous electrophoretic separation of a dye mixture (on the left) with the discontinuous, electrochromatographic separation of the same mixture (on the right) under identical conditions, after PUČAR[36]. Tropeolin O (T), fluorescein (F), chlorophenol red (P), rosolic acid (R_1 and R_2), chrysoidine Y (Ch) and nitraniline (N) in a borate buffer solution of pH 8.6 and ionic strength of 0.012. Filter paper, Munktell No. 20/150.

example, separated in $4M$ NH_4OH, travel on filter paper along the same paths[7] is no proof that these ions show, under these conditions, identical electrophoretic mobilities, especially as they are adsorbed onto the paper curtain to different degrees[73]. Accordingly, in continuous electrophoresis, adsorption on to the support, as well as the electrophoretic mobility, should be a factor in the end-result of continuous separation. This would then justify the expression "continuous electrochromatography"[7,50,74,75]. Recently ZWEIG[76] also sought to justify the expression "continuous electrochromatography" in that he reported a continuous separation of two dyes, brilliant blue and amaranth, of different adsorbabilities, on cellulose paper and glass fibre paper. Brilliant blue moved at pH 8.6 towards the cathode in the glass fibre paper, whilst on the cellulose paper it showed a weak anionic mobility. Amaranth moved, on the contrary, towards the anode in both supporting media. This example, however, demonstates no anomaly in the movement of brilliant blue, because glass fibre paper allows, in an alkaline medium, a much greater electroosmosis than cellulose paper[77]. The horizontal vector w' is, under the given conditions, much greater than it is on cellulose paper[77]. The improved continuous separation of both dyestuffs on glass fibre paper could be due to a smaller vertical buffer mobility w in the glass fibre paper, which under the same experimental conditions could lead to a relative increase in the angles of the paths.

3. *Blocking of the electrodes by a membrane*

If the paper curtain is separated by a membrane, for example a cellophane strip, from the electrode fluid, the horizontal or electrochromatographic, and also the vertical or

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chromatographic, components of the paths are, according to PUČAR[78], fundamentally altered. The cellophane membrane prevents to a large extent the horizontal capillary flow in the filter paper and the paper cannot then replace the loss of water due to Joule heat and consequent evaporation, by absorbing electrolyte solution from the electrode grooves. No gradients can arise in the electrolyte concentration in the horizontal direction, because the capillary electrolyte mobility in the horizontal direction is zero ($w' = 0$) and consequently the electric field intensity (H) in the horizontal direction is constant ($dH/ds = 0$).The vertical electrolyte mobility (W) is, contrary to the method discussed in the preceding section, certainly not constant, it decreases downwards due to evaporation and concentration of the electrolyte.

This decrease in the vertical mobility can evidently only be considered as a function of the electrolyte conductivity and the intensity of the electric field and is independent of the thickness of the filter paper. The angles of inclination of the tracks of the particles increase steadily downwards, as the tangent of the angle is $tg\alpha = uH/W$.

The downward increase in the inclination of the tracks is dependent on the increase in electrolyte conductivity and field intensity selected (Fig. 37).

Fig. 38 shows the vertical speeds of the dyestuff fronts of chlorophenol red, measured at intervals of 15 min, as a function of the time. Chlorophenol red does not

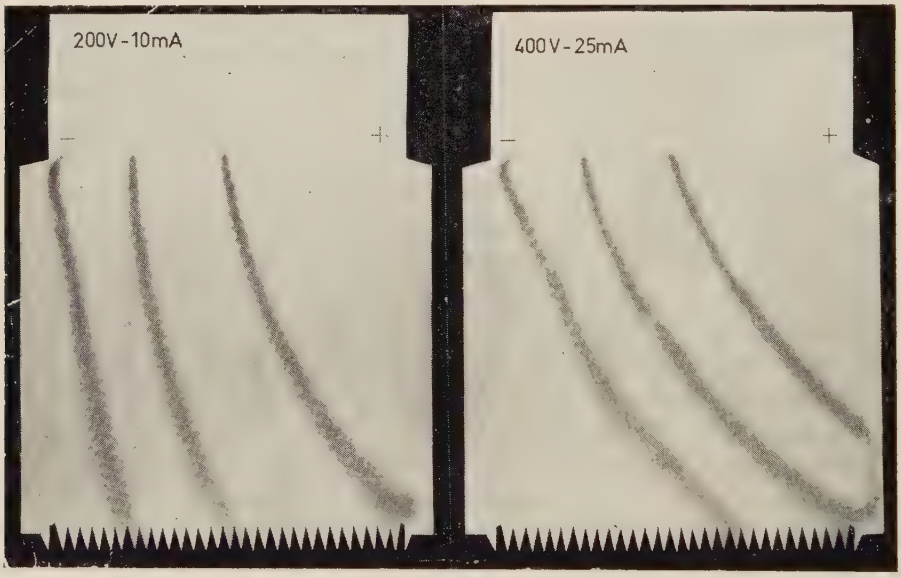

Fig. 37. Continuous electrophoresis of chlorophenol red in a boric acid–Na-acetate–NaOH buffer with blocking of the electrodes with a cellophane membrane, after PUČAR[78]. Buffer solution at pH 8.6 and ionic strength $\mu = 0.012$. Filter paper Munktell No. 20/150. The dyestuff solution was applied simultaneously at three different points of the apparatus. Left: 200 V and 10 mA. The flow time was 3h 45 min. Right: 400 V and 25 mA. The flow time was 4 h 15 min. The curves of the paths are bigger in the right-hand figure than in the left-hand one, because of the greater intensity of the electric field. The paths travel approximately parallel. Chlorophenol red is dissociated into two fractions: one dark red with greater mobility (clearly visible in the figure) and one yellow with a much lower mobility.

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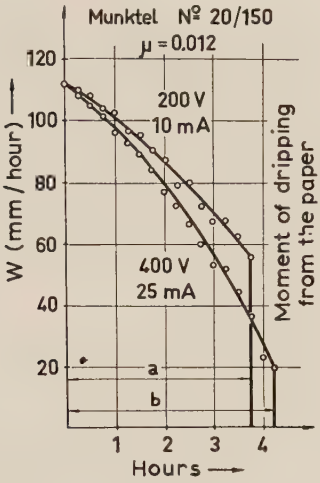

Fig. 38. The vertical component (W), according to PUČAR[78], as a function of the time and in relation to a particle subjected to electrophoresis. In the experiments illustrated in Fig. 37 the vertical speeds of the dyestuff fronts were measured every 15 min. "a" and "b" are the flow times for the experiments illustrated in Fig. 37 on the left and right.

Fig. 39. Continuous electrophoresis of a mixture of tropeolin O (T), fluorescein (F), chlorophenol red (P), rosolic acid (R_1 and R_2), chrysoidine Y (Ch) and nitraniline (N) with blocking with a membrane, after PUČAR[78]. All the experimental data as in the experiment on the right-hand side of Fig. 37. This experiment corresponds to the one illustrated on the left of Fig. 36. Because the point at which the substance was supplied was also the same, the two figures are directly comparable with one another. The flow times and current intensities were: without the membrane, 3 h 30 min and 20 mA (Fig. 36); and with the membrane blocking (Fig. 38) 4 h 15 min and 25 mA. Because of the greater curvature of the paths, all the fractions have travelled significantly further.

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show a chromatographic effect on filter paper ($R_F = 1$) under the given experimental conditions.

The distances "a" and "b" represent the corresponding flow times, that is the time elapsed between the admission of the substance into the apparatus and its exit from it. The further the particles travel downwards during this time the more the vertical velocity (*i.e.* the vertical component) decreases in the measure determined by the applied electric field and electrolyte conductivity and the greater the increase in the angle of inclination to the vertical of the tracks.

This explains the more pronounced curves of the tracks on the right side of Fig. 37 compared to those on the left.

In Fig. 39 the experiment shown on the left in Fig. 36 is repeated, but this time using a membrane. The flow time and current intensity have increased to a significant degree: without the membrane 3 h 30 min and 20 mA, and with the membrane 4 h 15 min and 25 mA. As the point of admission of the substance was identical, the two figures are directly comparable. In the second experiment all fractions travelled further due to the increased curvature of the tracks.

It is characteristic for continuous electrophoresis in a moist chamber with a membrane blocking the electrodes that, without exception all the paths show smaller or greater curvatures (depending on the conditions) in the sense of the apparent acceleration. It has the great advantage that the application of the membrane isolates completely the electrode grooves and prevents any loss in buffer, which is of decisive importance in the automatic washing of the electrodes in permanent set-ups for continuous work.

(*b*) *Terminology*

In the literature several terms have already been proposed for the different electrophoretic and electrochromatographic processes on filter paper and other supporting media, such as, for example, ionography[79], paper electrophoresis[36,71,72,80–83], paper ionophoresis[81], continuous electrophoresis[6,36,78,84,120–132], continuous ionophoresis[6], electrochromatography[7,36,50,74], continuous electrochromatography[7,21,50,74], zone electrophoresis[10], electrochromatophoresis[36,85], electromigration[86], etc. The terms for the individual processes are given in Table 1 according to a suggestion by PUČAR[36]. If there were general agreement in regard to the concept that adsorptive retardation is identical in the direction of the electric current and at right angles to it, then, as shown in the section on the vertical or chromatographic component, the term "continuous electrochromatography" would have to be abandoned, as the chromatographic component in the continuous process then has no influence on the separation. One-dimensional electrophoresis, or strip electrophoresis on porous supporting media is a process in which there is no chromatographic effect (all the fractions have $R_F = 1$, or $R_F \sim 1$), as, for example, in the usual paper electrophoresis of serum albumins and carbohydrates[87–89]. The one-dimensional separation of serum lipids is, on the other hand, an electrochromatographic process[88,89]. If the fractions show a noticeable chromatographic effect ($R_F < 1$), the term "strip electrophoresis"

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would only be applicable if the results were calculated, free from chromatographic effects, as for example, in the determination of electrophoretic mobilities[90–93].

The term "ionophoresis", which should emphasize the difference between the migration of ions and that of colloid particles, should, according to PUCAR[36], be avoided, because: (1) electrokinetically there is no essential difference; (2) it is often impossible to establish a sharp distinction between ions and colloid particles, especially in biological material; (3) the migration of colloid particles cannot be regarded as ionophoresis; it is, on the contrary, quite possible to regard the migration of ions as electrophoresis.

The term "two-dimensional electrochromatophoresis" or "two-dimensional electrochromatography" is not to be confused with electrochromatography in two directions, because electrochromatography in two (or several) directions, as described by DURRUM[94] or STRAIN[95], cannot be applied simultaneously in both directions. This technique is simply a combination of two one-dimensional processes. The same is also true of the combination of paper electrophoresis and paper chromatography as described by KICKHÖFEN AND WESTPHAL[96]. In this instance the technique is a combination of an electrophoretic or electrochromatographic process and a separate chromatographic process.

4. SOME EXAMPLES OF APPLICATIONS

(*a*) *Continuous electrophoretic separations*

1. *Mixtures of dyestuffs*

Dyestuffs were used by most authors for the investigation of the principles of the method and of their own apparatus. SVENSSON AND BRATTSTEN[4] and GRASSMANN[51] demonstrated the usefulness of their first trough-shaped separation chamber apparatus by the separation of bromophenol blue and bromothymol blue. GRASSMANN[51] was the first to describe the separation on filter paper of cresol red and bromophenol blue in *M*/15 phosphate buffer at pH 6.9. DURRUM[6] separated a solution of acid fuchsin in *N*/4 acetic acid into 7 visible components and 3 others visible in ultraviolet light. In the same electrolyte solution DURRUM[6] also separated acridine orange from a non-fluorescent red impurity, although both the components were very strongly adsorbed on filter paper. STRAIN AND SULLIVAN[7] separated methyl orange from phenolphthalein in 4*M* ammonia. GRASSMANN AND HÜBNER[119] separated some commercial acid and basic dyestuffs of the BASF, Bayer-Leverkusen, into their components. PUČAR[36,78] separated, in borate buffer at pH 8.6, a mixture of tropeolin O, fluorescein, chlorophenol red and rosolic acid, chrysoidine Y and nitraniline (see also Figs. 36 and 39). GRASSMANN AND HANNIG[20,33,38] described the separation of a synthetic mixture of bromophenol blue, bromocresol green, cresol red, fuchsin S and cresol purple in an acetic acid–pyridine buffer at pH 4.9 and the separation of a mono-, di-, tri- and tetra-sulphonic acid of a phthalocyanine in 1 *N* acetic acid[20]. DOBRY AND FINN[57] separated a mixture of basic fuchsin, bromothymol blue and bromophenol

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blue in acetate buffer at pH 5.3. BARROLLIER, WATZKE AND GIBIAN[60] separated, in 0.04 *M* ammonium acetate at pH 5.6, the mixture new coccine, bromophenol blue, uranine. ZWEIG[76] separated brilliant blue and amaranth in a veronal buffer at pH 8.6 with an ionic strength of 0.05.

2. Simple and compound proteins

The continuous separation of the coloured proteins phycoerythrin and phycocyanin, together with the separation of colourless serum proteins, accomplished somewhat later, may be considered as classical preparative separations of proteins by continuous electrophoresis. SVENSSON AND BRATTSTEN[4] separated a synthetic mixture of phycoerythrin and phycocyanin into its components in a phosphate buffer at a pH of 7.7 with ionic strengths of 0.025 and 0.050, and also separated natural extracts of the algae in which these proteins occur. The first separations of the serum proteins in a veronal-sodium, veronal-sodium–sodium acetate or phosphate buffer at pH 8.6 or 7.7 were reported by DURRUM[6], GRASSMANN AND HANNIG[5,34,51] and BRATTSTEN AND NILSSON[28]. In a series of subsequent publications BRATTSTEN separated in his pump apparatus large amounts of serum albumin[53,54,100] (see also Fig. 26), the algae proteins phycoerythrin and phycocyanin[54] and proteins from the cerebrospinal fluid[54] under various experimental conditions.

The toxins of a number of poisonous snakes were separated continuously in a veronal–sodium acetate buffer at pH 8.6 with an ionic strength of 0.05 by GRASSMANN AND HANNIG[97]. All the toxins investigated proved to be mixtures of a number of individual components, which were electrophoretically separable and of which some showed a definite enzyme action, such as, for example, protease, lecithinase, etc. GREGORY AND HOLDSWORTH[107] obtained by continuous electrophoresis in a 0.025 *M* veronal-sodium buffer at pH 8.6, a cyanocobalamin–protein complex in a pure state after addition of the vitamin to the milk of the pig, rat, goat, cow and to human milk.

OSTROWSKI AND NIEWIAROWSKA-PAWLUS[30] and SKARZYNSKI AND OSTROWSKI[108] purified, at pH 8.6, a vitamin B_{12}–protein complex (erythroglobulin) occurring in bovine serum and could concentrate the vitamin complex 625 times. ZAITLIN[109] separated continuously, in *M*/90 phosphate buffer at pH 6.9, an artificial mixture of two strains of the tobacco mosaic virus U_1 and U_2. He also[110] attempted a purification of the same virus under the same experimental conditions. TAKAHASHI, KARLER AND KNIGHT[111] described a purification of the infectious nucleic acid from tobacco mosaic virus in a phosphate buffer at pH 7.3.

BAMANN AND TIETZ[112] reported continuous separation of the lipase of the seeds of *Nigella sativa* in a veronal–sodium acetate buffer at pH 8.4. In the γ-globulin range two globulin fractions were obtained. PFEIL AND KANNGIESSER[113] obtained, in Michaelis buffer at pH 8.6 and after staining with ninhydrin, five visible fractions from a protein preparation from potato tubers. DOMBROWSKI[114] separated proteins from various carcinomata in a veronal–veronal-sodium buffer at pH 8.6 and prepared them in a pure state by dialysis and freeze-drying.

KARLER AND SPIVACK[115,116] used continuous electrophoresis very effectively for

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the purification of large quantities of the crude toxin of *Pasteurella pestis*. They found that repeated fractionation caused no loss of the toxin. KARLER[117] has also described a purification of tetanus toxoid, as well as a fractionation of complete homogenates of *Pseudomonas fluorescens*. In the latter instance 6 different enzymes were obtained and identified.

GRASSMANN, HANNIG AND SCHLEYER[118] described a continuous electrophoretic purification of a trypsin preparation in pyridine buffer at pH 6.1. A complete separation of the chymotrypsin which always accompanies the trypsin was obtained.

In addition to the authors already mentioned, numerous other investigators[20, 31, 33, 36, 38, 60, 62, 120–132, 143, 147] have carried out and described continuous separations of serum proteins. The protein fractions separated were either dialysed and freeze-dried, or immuno-chemically or analytically differentiated and identified, and tested for enzyme action within the individual fractions.

3. *Amino acids and peptides*

Already in the first years of continuous electrophoresis GRASSMANN AND HANNIG[5, 51] reported numerous continuous separations of various mixtures of amino acids. The following mixtures were separated: glutamic and aspartic acid in $M/100$ citrate buffer at pH 3.5 and $M/10$ citrate buffer at pH 3.79; glutamic acid–alanine–histidine in $M/15$ phosphate buffer at pH 5.2; lysine–histidine at pH 6.8; leucine–glutamic acid at pH 5.2; glycine–alanine at pH 11.5; alanine–threonine at pH 11.0; alanine–cysteine at pH 11.5; arginine–histidine–alanine–aspartic acid at pH 7.4; lysine–alanine–threonine–glutamic acid at pH 8.5; etc.

Fig. 40. Separation of human pathological serum (γ-plasmocytoma) in veronal buffer at pH 8.6, after PUČAR[36].

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DURRUM[6] described in 1951, a partial separation in 1 *N* acetic acid of a mixture of 9 amino acids: arginine–glycine–alanine–valine–serine–threonine–phenylalanine–proline-asparagine. The same year BRATTSTEN AND NILSSON[28] separated a mixture of glycine, alanine and glycylglycine in a formic acid buffer at pH 2.61. GRASSMANN *et al.*[34,98] continued after the work on amino acids with the separation of hydrolysates of casein, collagen, procollagen and insulin, for the identification and the quantitative determination of the amino acids. The first step should be a continuous group separation (Fig. 41) of the amino acids of the hydrolysates. Acetate

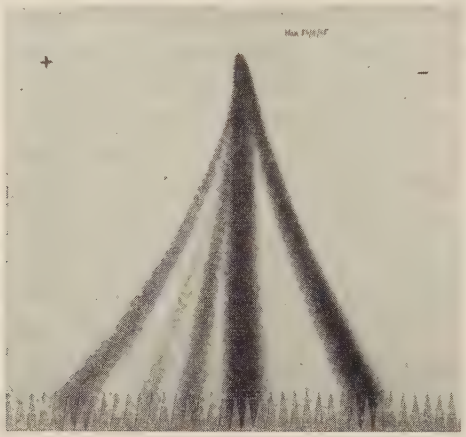

Fig. 41. Group separation of an albumin hydrolysate after GRASSMANN, HANNIG AND PLÖCKL[98]. Fraction B after performic acid oxidation of insulin. Pyridine–acetic acid buffer pH 3.9. Paper: Schleicher and Schüll 2040a, 290 V, 10 mA. From left to right, cysteic acid, aspartic acid, glutamic acid, neutral amino acids, basic amino acids.

buffer at pH 3.9 gives the best results. Under these conditions cysteic acid, aspartic acid and glutamic acid migrate completely separated from one another towards the anode, whilst all the neutral amino acids remain unseparated in a relatively narrow middle-to-cathode zone. The basic amino acids histidine, lysine and arginine move clearly separated from the others towards the cathode. The separation of the neutral amino acids is best done in 1 *N* acetic acid at pH 2.4. Fig. 42 shows the separation of 10 neutral amino acids: glycine, alanine, valine, leucine, serine, threonine, methionine, phenylalanine, proline and hydroxyproline. The separation of the basic amino acids histidine, lysine and arginine took place in *M*/25 borate buffer at pH 10.5 (Fig. 43).

Separation of peptides is carried out after GRASSMANN AND HANNIG[34] in 1 *N* acetic acid at pH 2.4. Mixtures of glycine, glycylglycine and diglycylglycine, as well as of L-leucyl-L-tyrosine, DL-leucylglycylglycine, glycine and diglycylglycine were completely separated. Also[34] a mixture resulting from the degradation of collagen by trypsin was continuously separated in *M*/20 acetate buffer at pH 5.0. Fig. 44 shows the separation of a mixture obtained by splitting procollagen with crystalline trypsin. According to GRASSMANN *et al.*[99], these are peptides of medium chain length made up of about 18 amino acids. The separations were carried out at pH 2.3, 4.9 and 9.2 on

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Schleicher and Schüll 604 L paper, treating 250 mg of the mixture during 24 h. GRASSMANN *et al.*[106] have also separated quantitatively (in 1 *N* formic acid) the fractions A and B of oxidised insulin obtained by decomposition with performic acid

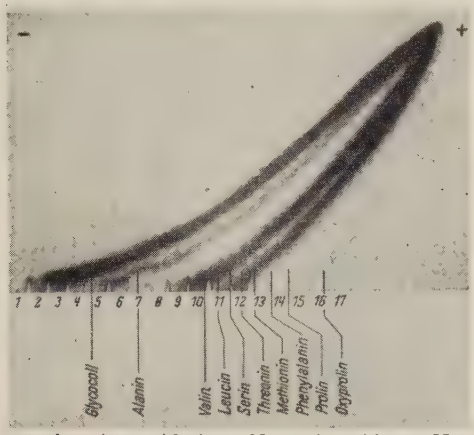

Fig. 42. Separation of neutral amino acids in 1 *N* acetic acid at pH 2.4 after GRASSMANN AND HANNIG[34]. Separation area 48 × 28 cm, Schleicher and Schüll 2040b, 700 V, 13 mA.

STEGEMANN[105] separated protein hydrolysates, containing basic, neutral, and acid amino acids on a PVC paper (Schleicher and Schüll, Rhovyl paper No. 1001) in 0.02 *M* phosphate buffer at pH 7.1. As buffer solvent a mixture of water and ethylene glycol monomethyl ether (Methylcellosolve) 3:7 is used, because it gives better wetting of the PVC paper.

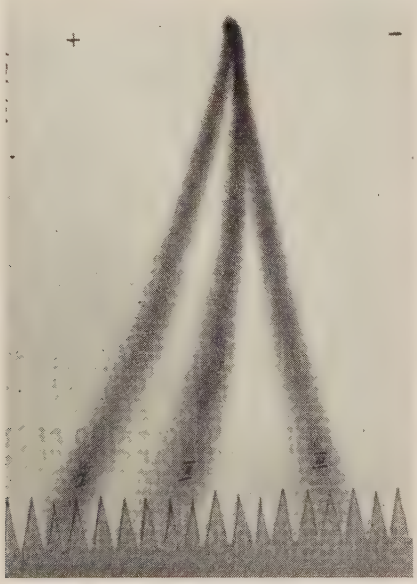

Fig. 43. Separation of basic amino acids in *M*/25 borate buffer at pH 10.5 after GRASSMANN AND HANNIG[34]. Separation area 23 × 22 cm, Schleicher and Schüll 2040b, 140 V, 10 mA. I, histidine; II, lysine; III, arginine.

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Continuous electrophoresis has been used for preparative separation of peptides obtained from crystallised human haemoglobin A by hydrolysis with trypsin. LIEBOLD *et al.*[133] used acetic acid (pH 2.5 – 2.6) as background electrolyte.

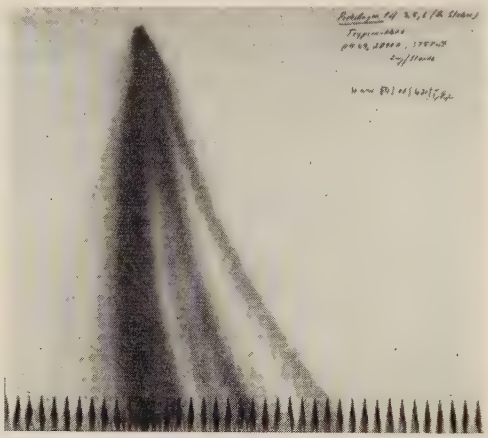

Fig. 44. Separation of a mixture of the products of decomposition of procollagen (peptides) by crystalline trypsin, in a pyridine–acetic acid buffer at pH 4.9, after GRASSMANN *et al.*[20,99], 375 V, 25 mA.

4. *Other separations of organic substances*

MICHEEL AND VAN DE KAMP[134] separated sugar mixtures in the form of boric acid complexes in $M/30$ boric acid, brought to pH 9.2 with NaOH. A mixture of cellobiose, L-rhamnose, D-mannose and D-glucose, and, also, an inulin hydrolysate consisting of D-fructose and D-glucose were continuously separated. MOULD AND SYNGE[135] separated as iodine complexes the products of hydrolysis of amylose with α-amylase. In a 0.04 N acetate buffer of pH 4.8 with 0.001 N I_2 and 0.002 N KI, three fractions were obtained, one blue, one red and one orange-yellow. GRASSMANN AND HANNIG[34] and GRASSMANN AND STADLER[136] separated continuously natural and synthetic tannins in $M/30$ acetate buffer at pH 5.0.

DECKERS AND SCHREIBER[137] carried out the separation of 100 mg of a mixture of the alkaloids scopolamine and hyoscyamine in a veronal buffer at pH 8.6 in 40 hours. WEISGLASS, PUČAR AND PETEK[138] separated continuously in 0.012 N ammonium acetate, ether and chloroform-extracts of a culture medium of *Pseudomonas pyocyanea*. Three pigmented fractions appeared, 8 fractions fluorescent in ultraviolet light and 7 that were colourless in normal and fluorescent light, but showed antibiotic activity. OSTROWSKI AND KRAWCZYK[139] purified by continuous electrophoresis in 0.5 N acetic acid a previously extracted vitamin B_{12} preparation. LEDERER AND MARINI-BETTOLO[16] separated, in 25 % acetic acid, the alkaloids from *Strychnos froesii*, which are fluorescent in ultraviolet light. UEDA AND MORI[146] separated preparations of the thyrotropic hormone in a veronal and phosphate buffer with pH of 8.0 to 8.6 and ionic strength of 0.05. The preparation Pretiron, made by Schering A.G., Berlin (18 mg), was

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separated continuously into three components and then the HCl-hydrolysates of the components were investigated by paper chromatography for individual sugars.

5. *Inorganic ions*

STRAIN AND SULLIVAN[7] were the first to report continuous separations of inorganic ions (1951). The following important separations were described. Ag–Cu in 0.1 *M* lactic acid or in a mixture of 4 *M* ammonia and 0.01 *M* EDTA; Ni–Cu and Ni–CrO_4^{--} in a mixture of 0.01 *M* disodium tartrate and 4 *M* ammonia; Fe–Al in a mixture of 0.005 *M* dimethylglyoxime, 0.01 *M* tartaric acid and 4 *M* ammonia; Sn–As–Sb in a mixture of 0.02 *M* lactic acid, 0.02 *M* tartaric acid and 0.04 *M* DL-alanine; Ag–Ni–Cu–Fe in a mixture of 0.01 *M* disodium tartrate, 0.01 *M* ammonium oxalate and 4 *M* ammonia; Ag–Ni in a mixture of 0.01 *M* ammonium oxalate and 4 *N* ammonia, or still better in 0.005 *M* EDTA in 4 *M* ammonia. In a later communication STRAIN[49] reported on further continuous separations, such as, for example: Co–Fe and Mg–Fe in a mixture of 0.05 *M* malonic acid and 0.1 *M* acetic acid, Tl–Al in a mixture of 0.05 *M* citric acid and 0.3 *M* ammonia, and Tl–As–Al and Ag–Cd–Bi–Al in a mixture of 0.05 *M* malonic acid and 0.05 *M* ammonia.

SANSONI AND KLEMENT[101] studied extensively the separation of condensed phosphates in a NaOH–Na-borate buffer at pH 10.0. A number of good quantitative separations of the condensed phosphates were reported. LEDERER[84] separated Y–La and Pr–Nd in 1 % citric acid. BIGHI AND TRABANELLI[102] separated the α,α'-dipyridyl complex of bivalent iron (Fe-Dip) from Fe^{++} and Fe^{+++}, or from Ni or Cr. The separations of Fe^{+++}–(Fe-Dip)–Fe^{++}, Ni–(Fe-Dip), Fe^{+++}–Ni–(Fe-Dip) and Cr–(Fe-Dip) were carried out in 1 % citric acid or 0.5 % or 1 % K-Na-tartrate. In addition separations of polythionates in 1 % K-Na-tartrate were reported, such as, for example, trithionate–tetrathionate and trithionate–pentathionate[102].

6. *Radioactive inorganic ions*

Continuous separations of radioactive inorganic ions were first reported by SATO, NORRIS AND STRAIN[50]. The following mixtures were separated: ^{45}Ca–$^{32}PO_4$ in 0.1 *M* lactic acid or in 0.1 *M* acetic acid[50], ^{90}Sr–^{90}Y in 0.1 *M* lactic acid[103], ^{90}Sr–^{90}Y–$^{32}PO_4$, ^{45}Ca–^{90}Y–$^{32}PO_4$, ^{90}Sr–^{90}Y–^{144}Ce–$^{32}PO_4$, ^{137}Cs–^{90}Sr–^{90}Y–^{60}Co–$^{32}PO_4$ and ^{137}Cs–^{90}Sr–^{90}Y–^{233}U–^{95}Zr–^{95}Nb in 0.1 *M* lactic acid[74]. LEDERER[84] separated Ac from inactive La. PUČAR AND JAKOVAC[104] separated two mixtures of rare earths, ^{144}Ce–^{160}Tb–^{170}Tm and ^{144}Ce–^{152}Eu–^{169}Yb in 0.05 *M* lactic acid (Fig. 45).

7. *Special applications*

According to GRASSMANN AND HÜBNER[119,141] the formation of loose addition compounds between two substances can be demonstrated in a simple fashion by the application of continuous electrophoresis. Contrary to the practice in normal separations, the two components are not admitted at the same point of the apparatus, but at two separate points so chosen that the migration paths of the two substances

with opposite charges cross each other. If no addition compounds are formed the two paths continue without interference with each other, as for example in Fig. 46 at the

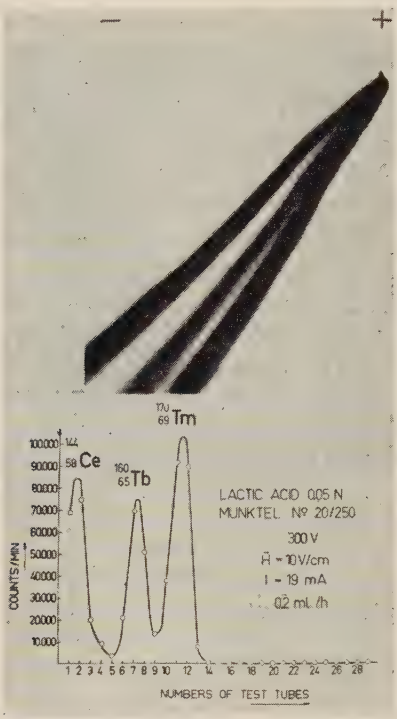

Fig. 45. Autoradiogram of a continuous electrophoretic separation of ^{144}Ce–^{160}Tb–^{170}Tm in 0.05*M* lactic acid after PUČAR AND JAKOVAC[104]. The diagram drawn below gives the activities in the collecting vessels, which were collected during a period of 0.5 h after establishment of the stationary state. Paper, Munktell No. 20/250.

left the dyestuff pair amido black and fuchsin. If, however, the formation of addition compounds takes place, as for example with orange II and methylene blue in Fig. 46 on the right, then the migration paths of the two components are deviated at the point of intersection and the complex formed, if it does not precipitate, moves in an

Fig. 46. The continuous crossing of two dyestuffs in 1 *N* acetic acid, after GRASSMANN AND HÜBNER[141]. Left: amido black–fuchsin. Right: orange II–methylene blue.

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intermediate direction. Later the two components separate again and continue in their original directions, but the dissociation of the complex results in the formation of ill-defined and fading tracks. NAKAMURA AND UETA[142] modified this method, in that instead of allowing the two solutions to flow continuously onto the paper, they traced directly on the paper with the two solutions, two rectilinear, crossing lines, similar to those shown in Fig. 46 on the left. The advantage of this method is that it is then possible to test, not only substances which move in opposite directions, but also those which move in the same direction. The enzyme-substrate combination of chymotrypsin and azocasein was demonstrated in this way. In many cases of biochemical or chemical significance, the formation of addition compounds in solutions, such as, for example, dyestuff–dyestuff, protein–dyestuff, protein–nucleic acid, antigen–antibody, enzyme–substrate, etc., will be shown by this method.

PUČAR, KELER AND PETEK[88] have also described these phenomena in the discontinuous and continuous electrophoresis of lipids from human sera (Fig. 47).

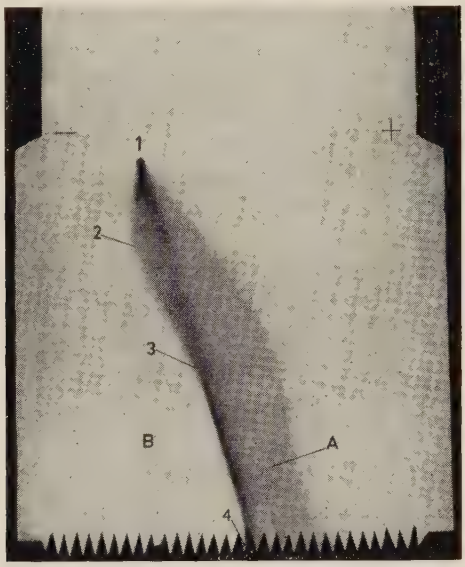

Fig. 47. Lipidogram of the continuous electrophoresis of a normal human serum in Na-veronal–Na-acetate buffer at pH 8.6, after PUČAR, KELER AND PETEK[88]. Filter paper Munktell No. 20/100. Staining with sudan black. 1 = point of entry of the serum; 2 = point of splitting of the γ-lipoprotein complexes into γ'-lipid and γ-globulin; 3 = point at which the formation of the γ'-lipid–α'_1-lipid complex occurs; 4 = point where the γ'-lipid–α'_1-lipid drips away; B = the paths (hardly visible) of the γ- and β-lipoproteins; A = A-lipoproteins; 1–2 = path of the γ'-lipoproteins; 2–3 = path of the free γ'-lipids; 1–3 = path of the free α'_1-lipids; 3–4 = path of the γ'-lipid–α'_1-lipid complex.

The γ'-lipids set free during the process of separation from γ-globulins suddenly changed their migration direction crossing the path of the α'_1-lipids and then travelled together with these to the exit points, probably in the form of a complex compound.

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(b) Discontinuous or two-dimensional electrochromatographic separations

Already in 1948 HAUGAARD AND KRONER[1] completely separated, by partition chromatography on paper with the simultaneous use of a potential gradient at right angles to the chromatographic direction, a mixture of 10 amino acids composed of two dicarboxylic acids (aspartic acid and glutamic acid), two basic amino acids (lysine and arginine) and six neutral amino acids (serine, glycine, alanine, valine, leucine and proline). It seems, nevertheless, as though this method has been little noticed and developed up till now. The separation sheet was pretreated with a phosphate buffer of pH 6.2 and phenol was used as solvent. BURMA[149] separated amino acid mixtures at pH 2.2, 4.0 and 6.0 by a similar technique.

STRAIN AND SULLIVAN[7] separated metal ions by the discontinuous method; some of these are: Ni–Co–Fe–Ag–Cd–Cu in a mixture of 0.01 *M* ammonium tartrate, 0.005 *M* dimethylglyoxime and 4 *M* ammonia; Hg–Bi–Cd–Pb–Cu in 0.1 *M* lactic acid and Ni–Fe–Co–Al in a mixture of 0.01 *M* tartaric acid, 0.005 *M* dimethylglyoxime and 4 *M* ammonia. ACHIWA[150–152] dealt with the separation of inorganic ions. The following separations, amongst others, were reported: SO_3^{2-}–SO_4^{2-}–CrO_4^{2-}–PO_4^{3-}–AsO_4^{3-} in a mixture of 0.0025 *M* $AgNO_3$ and 0.02 *M* ammonium acetate; S^{2-}–AsO_3^{3-}–BO_2^{-}–$Fe(CN)_6^{4-}$–$Fe(CN)_6^{3-}$ in a mixture of 0.005 *M* $Fe_2(SO_4)_3 \cdot (NH_4)_2SO_4$ and 0.1 *M* acetic acid; and I^-–Br^-–Cl^-–NCS^-–CN^- in a mixture of 0.01 *M* $NiSO_4$ and 0.02 *M* ammonium acetate. In addition a table was also given for the systematic qualitative analysis of all inorganic ions[150]. PUČAR AND JAKOVAC[104] separated, by the discontinuous

Fig. 48. Autoradiography of a two-dimensional electrochromatographic separation of ^{144}Ce–^{152}Eu–^{169}Yb in 0.05 *M* lactic acid, after PUČAR AND JAKOVAC[104]. Paper, Munktell No. 20/250. The horizontal broken line represents the front on which zones would be, if they were not adsorbed on filter paper.

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method, mixtures of the radioactive rare earths ^{144}Ce–^{160}Tb–^{170}Tm and ^{144}Ce–^{152}Eu–^{169}Yb in 0.05 *M* lactic acid (Fig. 48). PUČAR[36] separated (*cf.* Fig. 36) the dyestuff mixture tropeolin O–fluorescein–chlorophenol red–rosolic acid–chrysoidine Y–nitraniline, completely into its components in a borate buffer solution at pH 8.6. MCDONALD, BERMES AND SHEPHERD[42] separated in 14 min at pH 8.0 the dyestuff mixture aniline red–bromophenol blue–methyl orange with two-dimensional electrochromatography, accelerated by the use of centrifugal force. MCDONALD, RIBEIRO AND BANASZAK[45] separated in 15 min, by the same technique, in a phosphate buffer (pH 6.4, ionic strength 0.008) the amino acid mixture arginine–histidine–proline–tyrosine–aspartic acid. In a veronal buffer at pH 8.6 with an ionic strength of 0.01, MCDONALD AND RIBEIRO[44] separated the mixture glycine–lysine–glutamic acid.

PUČAR AND PETEK[87] separated proteins from normal and pathological human sera (Fig. 49). At pH 8.6 the protein fractions show slight adsorption on the cellulose of the filter paper, corresponding to the (adsorption) R_F value of 0.95, only α_2-globulin showing a smaller R_F value (0.89). All the protein fractions of the normal and pathological sera leave behind them visible tracks consisting of protein residues adsorbed on the filter paper. Intermediate tracks, which would correspond to mobilities lying between those of the chief protein fractions, could not be determined. By the same method PUČAR, KELER AND PETEK[88] showed that it is possible to separate and distinguish the non-adsorbed lipoproteins of serum from the lipids adsorbed on to the filter paper. On the basis of their chromatographic and electrophoretic behaviour the lipids of human sera were divided into three groups (Fig. 50): (1) the neutral fats (NF)

Fig. 49. Two-dimensional electrochromatogram of a pathological human serum (multiple myeloma) in a veronal–Na acetate buffer at pH 8.6, after PUČAR AND PETEK[87]. Stained for detection of the proteins. From right to left: albumin, α_1-, α_2-, β_1-, β_2-, γ_1-, γ_2-globulins. The tracks of the residual proteins are clearly visible.

firmly adsorbed at the starting point; (2) the free lipids, largely adsorbed on the filter paper, consisting of chylomicrons (chy) and a lipid fraction with the electrophoretic mobility of the α'-globulin (α'_1); and (3) the practically non-adsorbed protein-bound true lipoproteins. The carbohydrates, however, always travel with the corresponding protein fractions and cannot be separated from them by two-dimensional electrochromatography. On the basis of a great deal of clinical material, KELER-BAČOKA,

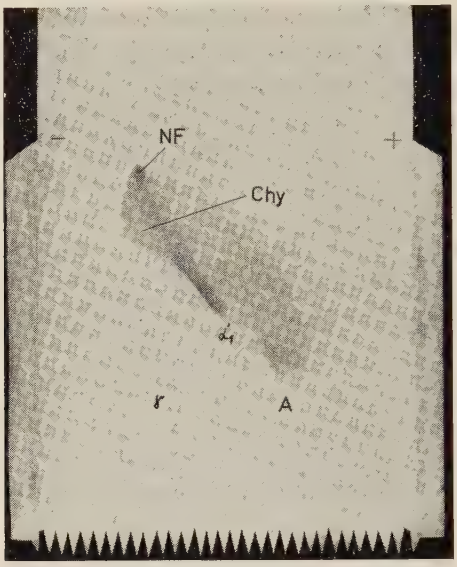

Fig. 50. Two-dimensional electrochromatogram of a normal human serum in a veronal–Na acetate buffer at pH 8.6, after PUČAR, KELER AND PETEK[88]. NF = neutral fats; Chy = chylomicrons, $\alpha'_1 = \alpha'_1$-lipids; A = A-lipoproteins; $\gamma = \gamma$-lipoproteins.

PUČAR AND PETEK[89] divide human sera, according to variations in their lipid structure, into one normal and five pathological lipid types. In conjunction with this BENAŠ, KELER-BAČOKA AND PUČAR[144] studied the action of heparin and of protamine sulphate on human serum lipids during alimentary hyperlipemia. The action of a colloidal solution of dextran on the lipids of human sera was demonstrated by the same technique by PUČAR, KELER-BAČOKA AND BENAŠ[145].

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SUPPLEMENTARY DATA FOR THE PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF PHENOL DERIVATIVES AND RELATED COMPOUNDS OF BIOCHEMICAL INTEREST, USING A "REFERENCE SYSTEM"

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I. INTRODUCTION

The present investigation should be regarded as a supplement to a previous series of investigations on phenolic derivatives and related compounds published in these Reviews[1]. In the earlier studies attention was mainly focused on substances found as metabolic products in mould cultures and on the possible application of this procedure to investigations within related fields.

A great number of closely related compounds was studied by paper chromatography in order to establish a systematic relationship between the structure and the R_F value, six different solvent systems and ten colour reagents being used in this investigation. The aim was to find a means of predicting, with a certain degree of probability, whether several functional groups occur in phenolic compounds when unknown mixtures or a desired type of compound have to be examined. It was found that there was a close connection between the structure and the different colour reactions, which followed certain rules, especially in the case of aromatic nuclei containing several hydroxy groups. Thus 1,2-, 1,3- and 1,4-dihydric phenol derivatives show significant characteristics when their colour reactions are compared with each other. From the R_F values of these three types of compounds in several solvent systems, it was possible at the same time to establish with certainty the presence of two hydroxy groups.

In order to extend to several types of compounds the investigations regarding the effect of various substituents on the colour and R_F value, some new data were collected and these are presented in this paper. These new data may be useful for the identification and description of aromatic compounds, not only those of vegetable origin, but also those occurring as metabolic products of living organisms, *e.g.* compounds originating from tryptophan, tyrosine and phenylalanine metabolism. Metabolic disorders have for many years been the object of intensive studies by several investigators and a great number of papers have appeared (for references see ARMSTRONG *et al.*[2], TOMPSETT[3], BOSCOTT AND BICKEL[4], and BOYLAND AND WILLIAMS[5]).

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The various mechanisms of detoxification of aromatic compounds, some of which are manifested by the excretion of hippuric acids, also belong to this subject and have been investigated independently or in connection with drug metabolism. Recently, interest has been centred on compounds with an indole structure, since it has been claimed that they are of significant importance in certain mental diseases, either because they are responsible to some extent for the condition or because they occur in the patients in greater amounts than are found in normal persons[6–9]. Further investigations in this direction would therefore be worthwhile. For this purpose the previous collection of compounds has been supplemented by several new types of compounds closely connected with the above-mentioned problems, and these were also submitted to paper-chromatographic analysis in several solvent systems.

2. MATERIALS AND METHODS

For one-dimensional descending chromatography rectangular glass jars of size 20 × 30 × 60 cm were used.

Whatman No. 1 filter paper with the dimensions 24 × 48 cm was employed.

The composition of the six solvent systems is given in the section "Abbreviations used in the Tables".

For spraying, the following ten standard reagents were used throughout the experiments:

Diazotized sulphanilic acid (obtained from Th. Schuchardt Co., Munich); diazotized 4-benzoylamino-2,5-dimethoxyaniline (Light Chemicals Co., England); diazotized *o*-dianisidine (Light Co.); *p*-nitrobenzenediazonium fluoborate (Eastman Kodak, U.S.A.); 2,6-dibromoquinone-4-chloroimide; 2,4-dinitrophenylhydrazine; ferric chloride; phosphomolybdic acid; potassium permanganate; bromophenol blue. For the composition of these reagents, see the section "Abbreviations used in the Tables".

Additional reagents: For indole derivatives the Ehrlich reagent[10–12], 1% solution of *p*-dimethylaminobenzaldehyde (abbreviated as EH) in 1 *N* HCl, was used. In order to obtain more distinctive shades and more strongly coloured spots varying from yellow to pink, blue and green, another similar reagent was introduced, which proved to be very useful although the background colour became more dominant after some time, owing to the oxidation of the reagent in the air. This reagent was prepared by dissolving *p*-dimethylaminocinnamaldehyde (obtained from Heidenheimer Chemisches Laboratorium, Heidenheim-Brenz, Germany) in 1 *N* HCl so that a 0.1% solution (abbreviated as DAC) was obtained. In most cases the coloured spots developed instantaneously. The use of 2-chloro-4-dimethylaminobenzaldehyde (Light Co.) in hydrochloric acid had no marked advantage over the classical Ehrlich reagent; in many cases it gave no reaction at all with indole derivatives.

In general these reagents gave yellow, red, violet, blue, green and grey colours with indole derivatives. However, alkaloids with an indole skeleton, such as reserpine, yohimbine, physostigmine, brucine and strychnine failed to react under the prevailing conditions. Also some other types of compounds gave colours, *e.g.* urea (EH – yellow,

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DAC – carmine), thyroxine (EH – yellow, DAC – magenta), kynurenine (EH – orange, DAC – carmine), histidine (EH – yellow, DAC – nil), *p*-aminobenzoylglutamic acid (EH – orange, DAC – red violet), 2,5-diaminobenzoic acid (EH – carmine, DAC – blue), N-*p*-hydroxyphenylglycine (EH – yellow, DAC – magenta), N-*o*-carboxyphenylglycine (EH – orange, DAC – carmine), formylglycine (EH – yellow, DAC – orange), ureidosuccinic acid (EH – yellow, DAC – pink), histamine (EH – nil, DAC – orange), sarcosine (EH – nil, DAC – orange), hydantoin (EH – nil, DAC – carmine), hydantoic acid (EH – yellow, DAC – carmine), allantoin (EH – pale yellow, DAC – yellow) and allantoic acid (EH – pale yellow, DAC – carmine), and *m*- and *p*-nitrohippuric, 3,5-dinitrohippuric and *p*-aminohippuric acids (EH – yellow, DAC – carmine).

For detecting hippuric acid derivatives a 2% solution of recrystallized *p*-dimethylaminobenzaldehyde (abbreviated as DAB) in acetic anhydride was used[13,14]. This reagent gave yellow, orange and pink colours with hippuric acid derivatives; some of these derivatives, however, gave weak shades. On the other hand, closely related compounds such as formylglycine, α-aminophenylacetic acid, N-*o*-carboxyphenylglycine, *p*-aminobenzoylglutamic acid, creatinine, betaine and hydantoic acid gave yellow colours. Compounds such as phenoxyaceturic acid, phenaceturic acid, N-phenacetylglutamic acid gave no colour reaction.

Even compounds like *o*-hydroxyphenylacetic acid and homogentisic acid show yellow colours with DAB reagent after 24 hours.

As noticed earlier by OSTEUX AND LATURAZE[15], the hippuric acid reagent gave reddish colours with some aliphatic compounds such as *cis*- and *trans*-aconitic acid, aconitic anhydride. In order to establish the possible disturbances that might arise from other compounds than hippuric acid derivatives, the aliphatic compounds listed in Tables 18 to 21 in the previous publication[1] and Table 15 in the present paper, were tested. Weak yellow colours that could hardly be distinguished from the background colour at this concentration level were observed with the following compounds: malic acid, fumaric acid, itaconic acid, mesaconic acid, pyruvic acid, β-ketoglutaric acid, citric acid and mesoxalic acid. The spots were, however, more discernible in U.V. light.

In an attempt to obtain clearer differences in colour between the hippuric acid derivatives a 1% solution of *p*-dimethylaminocinnamaldehyde and a 2% solution of 2-chloro-4-dimethylaminobenzaldehyde in acetic anhydride were also tested. The former was shown to be fairly reactive, giving stronger red-brownish colours, but it was not so easy to distinguish the shades from each other. The reagent prepared from the chloro-derivative did not show any advantage over the above-mentioned reagents as a detecting agent for hippuric acids.

For detecting alkaloids, a Dragendorff reagent[16–18] was prepared by dissolving 2 g $KI \cdot BiI_3$ (obtained from Merck Co., Darmstadt) in 100 ml 0.01 *N* HCl and filtering the hot solution. This reagent gave orange colours with alkaloids, and it proved possible to detect the latter when the other reagents failed to react.

In general the paper-chromatographic procedure followed essentially the same principles as described earlier[1]. The colours obtained with the different reagents were

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recorded as numbers, following the "Derwent" colour pencils index of Cumberland Pencil Co., Keswick, England. The description of these colours is found under the heading "Colour Index for the Tables".

The compounds investigated were mainly obtained from commercially available preparations. The hydroxy-derivatives of phenyllactic and phenylpyruvic acids were synthetized by Dr. B. WENGLE in this laboratory, to whom the author is greatly indebted.

The various hippuric acid derivatives used in this investigation were synthetized by the author, mainly according to two methods and on a scale of *ca.* 10 mmole.

Synthesis of hippuric acid derivatives

(1) Substituted non-hydroxylated hippuric acid derivatives were in general synthetized according to the procedure described by NOVELLO *et al.*[19] for *o*-chlorohippuric acid, by shaking a glycine solution in sodium hydroxide with the corresponding benzoyl chloride and recrystallizing the product from a minimum amount of hot water.

Ortho-, *meta-* and *para*-hydroxy-hippuric acids were prepared according to FISCHER[20], the OH-group of the phenolic acid being first protected by treatment with chloroformic acid ethyl ester, which yields an O-carbethoxy derivative. The corresponding acid chloride was subsequently obtained by treating this derivative with phosphorus pentachloride. The substituted benzoyl chloride was then condensed in ether with an excess of glycine ethyl ester. The precipitated glycine ester hydrochloride was removed and the residue was hydrolysed with 1 *N* sodium hydroxide; the hippuric acid derivative was isolated after acidification and was finally recrystallized from water.

(2) The second type of synthesis was carried out according to SHEEHAN AND HESS[21], and ITO AND NEILANDS[22], whereby equimolar amounts of the corresponding hydroxy- or dihydroxy-benzoic acid and glycine ethyl ester (obtained by treating glycine ethyl ester hydrochloride with silver oxide in tetrahydrofuran and filtering off silver chloride) in tetrahydrofuran were condensed with dicyclohexyl-carbodiimide in slight excess, under nitrogen atmosphere, overnight at room temperature. Acetic acid was added to decompose the condensing agent. Urea was removed and the solvent was evaporated to dryness. The ester residue was hydrolysed with 1 *N* NaOH at room temperature for four hours, and the product was isolated, after acidification, by extraction with ethyl acetate. It was recrystallized in a minimum amount of hot water. The yields were about 50% in the case of *o*-hydroxyphenylacetylglycine.

This method has proved suitable and more convenient for hydroxylated derivatives, since the steps of protecting the hydroxy groups by converting them into O-carbethoxy groups is eliminated. Especially when synthetizing dihydroxy-derivatives the protection of both OH-groups by chloroformic acid ethyl ester is rather tedious, since usually only one group can be protected at a time (see FISCHER[23]).

α-Hydroxyhippuric acid was synthetized according to HAAS[24], whereby hippuric

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acid, suspended in carbon tetrachloride, was oxidized with bromine in the presence of red phosphorus. The product was recrystallized several times from water.

It is intended to publish more detailed reports on the syntheses of various hippuric acids, as well as data on the occurrence of hippuric acids in urine.

3. GUIDE TO TABLES 1–15

The following Tables present information compiled for approx. 270 organic compounds, investigated by the procedure outlined above. The R_F values are recorded in six different solvent systems designated by F, E, A, B, C and D. For the composition of these solvents, see the list of abbreviations given below. Under the heading "Detection" (columns 2–11) the colour reactions are recorded for 10 different reagents used for the identification of each compound; in addition, the colour produced in ultraviolet light is indicated in the first column under this heading. The amount of substance used in these experiments was 25–50 γ per spot, which produced different colours when the reagents were applied. These colours are referred to by numbers, the explanation of which is found in the colour index. For abbreviations and the composition of the reagents, the reader is referred to the following section.

Table 1 Monohydric phenols and their derivatives.
Table 2 1,2-Dihydric phenols and their derivatives.
Table 3 1,3-Dihydric phenols and their derivatives.
Table 4 1,4-Dihydric phenols and their derivatives.
Table 5 Trihydric phenols.
Table 6 Naphthalene and quinoline derivatives.
Table 7 Benzoic and phthalic acid derivatives.
Table 8 Aromatic non-phenolic and heterocyclic monocarboxylic acids with the COOH group in the side chain, and their derivatives.
Table 9 Pyridinedicarboxylic acids, non-hydroxylated.
Table 10 Aromatic, heterocyclic and some aliphatic amino acid derivatives.
Table 11 Biologically active nitrogen compounds, *e.g.* biogenic amines, etc.
Table 12 Indole derivatives.
Table 13 Natural products of vegetable and mould origin, *e.g.* alkaloids, antibiotics, etc.
Table 14 Hippuric acid derivatives.
Table 15 Miscellaneous aliphatic carboxylic acids.

ABBREVIATIONS USED IN TABLES 1–15

Chromatographic solvent systems:

F = Ethyl methyl ketone–acetone–formic acid–water (40:2:1:6 parts by volume)
E = Ethyl methyl ketone–diethylamine–water (921:2:77 parts by volume)
A = Methyl isobutyl ketone–formic acid–water (10 parts ketone saturated with 1 part 4% formic acid)
B = Chloroform–methanol–formic acid–water (10 parts of chloroform saturated with a mixture of 1 part methanol and 1 part 4% formic acid)
C = Benzene–ethyl methyl ketone–formic acid–water (a mixture of 9 parts benzene and 1 part ketone saturated with 1 part 2% formic acid)
D = Benzene–formic acid–water (10 parts benzene saturated with 1 part 2% formic acid)

Reagents used for detection:

U.V. = Ultraviolet light
D1 = Diazotized sulphanilic acid (0.3% solution in dioxane–water 1:2)
D2 = Diazotized 4-benzoylamino-2,5-dimethoxyaniline (0.6% solution in dioxane–water 1:2)
D3 = Diazotized *o*-dianisidine (0.6% solution in dioxane–water 1:2)
D4 = *p*-Nitrobenzenediazonium fluoborate (0.4% solution in dioxane–water 1:2)
DB = 2,6-Dibromoquinone-4-chloroimide (0.5% solution in dioxane–acetone 4:1)
DN = 2,4-Dinitrophenylhydrazine (*ca.* 0.1% solution in 1 *N* HCl)
Fe = Ferric chloride (2% aqueous solution)
Mo = Phosphomolybdic acid (2% aqueous solution)
Mn = Potassium permanganate (1% aqueous solution)
Ind = Bromophenol blue (*ca.* 0.05% solution in ethanol)

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Special reagents used for detection:

NH = Ninhydrin reagent (2% solution in butanol saturated with water)
EH = Ehrlich reagent (1% *p*-dimethylaminobenzaldehyde in 1 *N* HCl)
DAC = *p*-Dimethylaminocinnamaldehyde (0.1% solution in 1 *N* HCl)
DAB = *p*-Dimethylaminobenzaldehyde (2% solution in acetic anhydride)
Bi = Dragendorff reagent (2% solution of potassium bismuth tetraiodide in 0.01 *N* HCl)

Owing to the lack of space in the tables, the R_F values have been recorded as 12, 56, 88, but should be read: 0.12, 0.56, 0.88 etc. The colours produced by the reagent are recorded as numbers, and the corresponding shades can be found in the colour index. The —sign means that no reaction was observed under the prevailing conditions. The +sign indicates an uncertain reaction, which was too weak to deserve colour estimation. Reactions with the reagents Mn and Ind are only indicated by the signs: —, + or ++. The + +sign means that a positive reaction was obtained immediately. In the few cases where the colours are recorded by a number placed on top of another number, *e.g.* $\left.\begin{matrix}24\\57\end{matrix}\right\}$, this indicates that immediately upon spraying a violet spot (24 in the colour index) appears which, within a few seconds, turns to light brown (57 in the colour index). Usually most colours are unstable and after some time take on a brownish tone, which is to some extent caused by the chemical influence of other reagents used in the vicinity. This change in colour is not recorded in the tables, nor is there any column for those compounds that, at this low concentration, are visible on the chromatograms because of their own colour.

The various compounds listed in the tables have been arranged as far as possible according to the following principles:

(1) Free phenols together with their methoxy- and alkyl-derivatives; (2) the corresponding amino- and nitro-derivatives; and (3) further derivatives in the order: hydroxymethyl, keto, aldehyde and finally monocarboxylic acid derivatives, derivatives with a carboxylic acid group in the side chain and dicarboxylic acids.

COLOUR INDEX FOR THE TABLES

The colours that were produced by the action of different reagents on the investigated compounds listed in Tables 1–15, have been recorded as numbers according to the following code:

01	Zinc Yellow	25	Dark Violet	49	Sap Green
02	Lemon Cadmium	26	Light Violet	50	Cedar Green
03	Gold	27	Blue Violet Lake	51	Olive Green
04	Primrose Yellow	28	Delft Blue	52	Bronze
05	Straw Yellow	29	Ultramarine	53	Sepia
06	Deep Cadmium	30	Smalt Blue	54	Burnt Umber
07	Naples Yellow	31	Cobalt Blue	55	Vandyke Brown
08	Middle Chrome	32	Spectrum Blue	56	Raw Umber
09	Deep Chrome	33	Light Blue	57	Brown Ochre
10	Orange Chrome	34	Sky Blue	58	Raw Sienna
11	Spectrum Orange	35	Prussian Blue	59	Golden Brown
12	Scarlet Lake	36	Indigo	60	Burnt Yellow Ochre
13	Pale Vermilion	37	Oriental Blue	61	Copper Beech
14	Deep Vermilion	38	Kingfisher Blue	62	Burnt Sienna
15	Geranium Lake	39	Turquoise Blue	63	Venetian Red
16	Flesh Pink	40	Turquoise Green	64	Terra Cotta
17	Pink Madder Lake	41	Jade Green	65	Burnt Carmine
18	Rose Pink	42	Juniper Green	66	Chocolate
19	Madder Carmine	43	Bottle Green	67	Ivory Black
20	Crimson Lake	44	Water Green	68	Blue Grey
21	Rose Madder Lake	45	Mineral Green	69	Gunmetal
22	Magenta	46	Emerald Green	70	French Grey
23	Imperial Purple	47	Grass Green	71	Silver Grey
24	Red Violet Lake	48	May Green	72	White=colourless

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TABLE I

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME MONOHYDRIC PHENOLS AND THEIR DERIVATIVES

R_F values × 100						Compounds	Detection										
F	E	A	B	C	D		U.V.	D1	D2	D3	D4	DB	DN	Fe	Mo	Mn	Ind
90	93	92	92	93	92	2,3,6-Trimethylphenol	—	09	15	63| 64|	29| 25|	35	—	—	68	++	—
95	91	89	93	92	92	2,4,6-Trimethylphenol = mesitol	—	60	60	17	06	30	—	—	30| 68|	++	—
92	92	93	92	91	87	3,4,5-Trimethylphenol	—	08	08	64	62	—	—	—	71	+	—
95	90	92	92	93	92	2,6-Dibromo-4-*tert.*-butylphenol	—	08	62	60	59	+	—	—	71	—	—
95	93	92	90	89	92	2,4-Di-*tert.*-butylphenol	—	07	09	62	09	—	—	—	71	+	—
94	95	96	93	94	93	2,4,6-Tri-*tert.*-butylphenol	—	—	—	—	—	—	—	—	—	+	—
93	91	88	92	82	89	Dichlorophene = *o*-methylene-bis-*p*-chlorophenol	—	11	58	65	09	31| 35|	—	—	69	++	—
37	93	01	01	00	00	4-Chloro-*o*-anisidine	56	—	—	—	—	—	—	—	08	+	—
87	83	86	76	90	92	2-Chloro-4,6-dinitrophenol	56	—	—	+	—	—	—	+	—	+	++
93	93	96	96	95	95	5-Methoxyindole	71	03	+	+	10| 62|	24	—	—	51| 69|	+	—
94	95	94	96	96	96	5-Benzyloxyindole	—	—	—	—	09	23	—	—	71	+	—
94	91	96	92	93	89	*o*-Methoxybenzyl alcohol	—	—	—	—	—	—	—	—	—	+	—
84	83	84	37	22	03	*m*-Hydroxybenzyl alcohol	—	09	12	15	08	35	—	—	71	++	—
94	86	93	94	88	91	*m*-Methoxybenzyl alcohol	—	—	—	—	—	—	—	—	—	+	—
85	82	80	29	18	02	*p*-Hydroxybenzyl alcohol	—	08	10	15	07	35	—	—	71	++	—
91	88	84	87	83	81	*p*-Methoxybenzyl alcohol	—	—	—	—	—	—	—	—	—	+	—
94	92	92	88	87	86	2-Hydroxy-5-methylacetophenone	17	+	—	—	—	—	+	26| 24|	—	+	—
92	84	92	87	84	57	4-Hydroxy-3-methylacetophenone	—	09	—	+	06	—	57	—	—	+	—
95	93	92	89	90	90	*o*-Hydroxypropiophenone	—	07	09	+	+	40| 23|	+	24	—	+	—
93	90	92	89	87	68	*m*-Hydroxypropiophenone	—	09| 13|	60	60	11	39	+	—	—	+	—
90	87	84	86	79	49	5-Methoxy-3-indolealdehyde	34	—	—	—	—	—	08	—	71	+	—
92	18	89	86	77	45	Phenoxyacetic acid	—	—	—	—	—	—	—	58	—	—	++
85	05	69	25	09	00	*p*-Hydroxyphenoxyacetic acid	—	19	09	23| 63|	08| 25|	32| 12|	—	—	30	++	+
87	07	85	88	84	78	*o*-Methoxybenzoic acid	34	—	—	—	—	—	—	03	—	—	+
94	13	96	93	93	91	*m*-Methoxybenzoic acid	—	—	—	—	—	—	—	—	—	—	+
93	66	94	90	89	74	3-Phenylsalicylic acid	38	10	60	65	09	38	—	24| 68|	—	++	++
90	34	93	61	66	08	5-Formylsalicylic acid	—	—	—	—	—	—	06	65	—	+	+
92	72	92	78	85	51	3,5-Dichlorosalicylic acid	38	07	57	64	06	—	—	23	—	++	++
93	78	91	85	87	64	3,5-Diiodosalicylic acid	69	—	—	—	—	—	—	24	—	+	++
84	63	79	42	46	06	3-Nitrosalicylic acid	56	—	—	—	—	03	—	63	06	—	+
93	04	90	73	66	04	3-Chloro-4-hydroxybenzoic acid	—	07	—	65	64	39	—	03	—	++	+
93	00	95	88	82	43	3-Nitro-4-hydroxybenzoic acid	56	07	07	—	12	—	—	57	09	+	+
93	73	95	93	91	85	*o*-Thymotic acid	38	12	08	56	08| 23|	29| 25|	—	70	56| 71|	+	+
89	08	83	39	19	00	3-Hydroxyanthranilic acid	38	58	57	62	63	42	—	59	51| 68|	+	+
89	04	90	53	38	02	*m*-Hydroxycinnamic acid	—	—	+	63	08| 20|	38	—	—	—	++	+
90	10	92	91	83	70	*p*-Methoxycinnamic acid	38	—	—	—	—	—	—	—	—	+	+
93	19	96	87	78	22	4-Hydroxy-α-phenylcinnamic acid	—	12	57	56| 63|	23	—	—	57	50| 71|	++	+
88	23	92	51	62	02	4,4′-Bis-(4-hydroxyphenyl)-pentanoic acid	—	09	11	15	20	38	—	—	69	++	+
88	08	93	80	58	12	5-Methoxy-2-carboxy-indole	33	—	+| 25|	24	+	—	—	59	—	—	+

(continued on p. 99)

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TABLE 1 (*continued*)

R_F values × 100						Compounds	Detection										
F	*E*	*A*	*B*	*C*	*D*		*U.V.*	*D1*	*D2*	*D3*	*D4*	*DB*	*DN*	*Fe*	*Mo*	*Mn*	*Ind*
90	32	92	55	42	03	*o*-Hydroxyphenylacetic acid	34	12	14	65 63	08	29	—	71	51 30	+ +	+
88	05	86	48	35	02	*p*-Hydroxyphenylacetic acid	—	14	58	65	65 60	—	—	—	30	+ +	+
92	27	86	71	63	16	*o*-Hydroxyhydrocinnamic acid = melilotic acid	—	12	13	65	08	29	—	—	45	+ +	+
89	09	89	48	29	01	*p*-Hydroxycinnamic acid	38	07	12	65	08	26	—	60	30	+ +	+
87	22	78	31	13	00	*o*-Hydroxyphenyllactic acid	—	06	—	65	09	33	—	—	—	+	+
88	28	82	32	27	02	*o*-Hydroxyphenylpyruvic acid	34	08	09	65 62	09	41	06	48	71	+	+ +
90	19	80	11	08	01	*m*-Hydroxyphenylpyruvic acid	70	07	—	25 59	07	57	06	45	—	+	+
52	40	16	54	07	05	2-Hydroxypyridine	—	—	—	—	—	—	—	09	—	+	—
42	57	28	41	03	00	3-Hydroxypyridine	34	08	63 65	23	60 09	29	—	09	—	+ +	—
23	08	01	03	00	00	4-Hydroxypyridine	34	—	—	—	—	—	—	07	—	—	—

TABLE 2

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME 1,2-DIHYDRIC PHENOLS AND THEIR DERIVATIVES

R_F values × 100						Compounds	Detection										
F	*E*	*A*	*B*	*C*	*D*		*U.V.*	*D1*	*D2*	*D3*	*D4*	*DB*	*DN*	*Fe*	*Mo*	*Mn*	*Ind*
91	83	92	67	74	22	4-Chloropyrocatechol	—	10 14	14 64	17	56 65	29	—	42	68	+ +	—
93	92	91	89	89	74	3-Isopropylpyrocatechol	56	10 64	24	23	10 65	23	—	54 67	62 69	+ +	—
92	93	92	90	87	69	4-*tert.*-Butylpyrocatechol	56	05 60	27	27 71	08 64	24 28	—	03 48	62 25	+ +	—
92	93	90	92	89	87	3-Isopropyl-6-methylpyrocatechol	56	10 64	65 66	61	10 65	28 25	—	60	52 69	+ +	—
94	93	95	90	92	87	3,5-Diisopropylpyrocatechol	56	06 70	58 41	58 39	59	41 27	17	03	69 42	+ +	—
93	78	84	92	72	58	Veratryl alcohol	—	—	—	—	—	—	—	—	—	+	—
86	88	85	91	82	69	*m*-Acetovanillone	—	19	60	65	21 65	38	07	—	68	+ +	—
88	90	87	90	87	85	Zingerone = vanillyl-acetone	—	19	60	62	60 55	40	06	40 51	30	+ +	—
28	63	00	00	00	00	Vanillylamine	—	10	15	19	56 23	35	—	—	68	+	—
92	62	87	92	85	87	Ethylvanillin = 3-ethoxy-4-hydroxybenzaldehyde	—	09	—	61	59	+	08	—	71	+ +	—
94	79	93	92	93	90	5-Propenylvanillin	34	09	—	—	+	51	08	—	71	+ +	—
88	07	83	83	62	22	Opianic acid = 5,6-dimethoxy-2-formylbenzoic acid	26	—	—	—	—	—	03	—	—	+	+
92	14	86	88	80	02	Piperonylic acid	34	—	—	—	—	—	—	58	—	+	+
82	02	66	07	01	00	3,4-Dihydroxyphenylacetic acid	—	11	20	22	62	24	—	43	62 69	+ +	+
89	08	92	83	69	38	3-Ethoxy-4-hydroxycinnamic acid	38	65	23	25 54	38 43	25	—	57	69	+ +	+
91	12	90	90	89	67	2,3-Dimethoxycinnamic acid	32	—	—	—	—	—	—	—	—	+	+
82	00	69	45	04	00	Hemipinic acid	+	—	—	—	—	—	—	03	—	—	+

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TABLE 3

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME 1,3-DIHYDRIC PHENOLS AND THEIR DERIVATIVES

R_F values × 100						Compounds	Detection										
F	E	A	B	C	D		U.V.	D1	D2	D3	D4	DB	DN	Fe	Mo	Mn	Ind
94	67	92	72	75	33	2,6-Dichlororesorcinol	—	09	64	65	10	23	—	70	68	++	—
93	92	92	87	91	82	2,5-Dimethylresorcinol	—	06	19	65	08	33	—	—	—	+	—
95	93	92	91	89	83	Dimethoxyorcinol	—	06	19	65	08	33	—	—	—	+	—
41	77	08	02	00	00	2-Aminoresorcinol	—	08 60	19	63	09	24	—	56	68	++	—
93	47	92	90	91	92	2-Nitroresorcinol	56	06 65	15	65	08	23	—	—	51	+	—
93	78	94	91	90	78	2,4-Dihydroxybenzophenone	71	64	20 63	23 25	11	28	+	62	05	+	—
94	93	95	92	93	93	2-Hydroxy-4-methoxybenzophenone	65	09	+	63	08	—	+	58	—	+	—
91	49	93	85	84	46	Orsellinic aldehyde	17	08	19	23	08	69	08	63	—	+	—
90	04	84	87	74	54	2,6-Dimethoxybenzoic acid	—	—	—	—	—	—	—	—	—	—	+
92	17	92	92	85	72	3,5-Dimethoxybenzoic acid	34	—	—	—	—	—	—	—	—	—	+
93	19	93	90	86	76	2,4-Dimethoxycinnamic acid	34	—	—	—	—	—	—	57	—	+	+

TABLE 4

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME 1,4-DIHYDRIC PHENOLS AND THEIR DERIVATIVES

R_F values × 100						Compounds	Detection										
F	E	A	B	C	D		U.V.	D1	D2	D3	D4	DB	DN	Fe	Mo	Mn	Ind
86	dec.*	86	43	33	08	Methoxyquinol	34	52	56	51	10	66	—	60	49	++	—
78	00	65	32	21	08	2,5-Dihydroxy-1,4-benzoquinone	65	08	22	65	10	26	58	56	71	++	+
39	00	07	06	00	00	3,6-Dihydroxypyridazine	—	—	—	—	—	—	—	17	—	+	—
92	68	89	73	51	08	*p*-Dihydroxypropiophenone	34	58	63	—	61	52	58	43	09 68	++	—
92	92	95	94	92	93	2,5-Dimethoxybenzaldehyde	39	—	—	—	—	—	07	—	—	+	—
84	dec.*	71	03	02	00	Homogentisic acid	34	57	70	72	58	70	—	—	30 68	++	+
90	dec.*	90	78	71	26	Homogentisic lactone	+	24 52	62 69	15 27	63	23 45	—	—	25 68	++	—
95	12	94	92	92	91	2,5-Dimethoxyphenylacetic acid	—	—	—	—	—	—	—	—	—	+	+

* Decomposition.

4. RESULTS AND DISCUSSION

The first five tables (1–5) present chromatographic data on monohydric, 1,2-, 1,3-, 1,4-dihydric and trihydric phenols. The grouping into tables was the same as that previously used, in order to demonstrate the different colour reaction patterns, which are in accordance with earlier findings as far as the three groups of dihydric phenol derivatives are concerned. The 1,3-dihydric phenol derivatives show a very distinct colour pattern when the four diazonium reagents are applied, except when the OH-groups are methoxylated and, in addition, there is a carboxyl group present.

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TABLE 5

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME TRIHYDRIC PHENOLS

R_F values × 100						Compounds	Detection									
F	E	A	B	C	D		U.V.	D1	D2	D3	D4	DB	DN	Fe	Mo	Mn	Ind
85	83	77	02	01	00	2,4,6-Trihydroxytoluene	+	10	63	65	10	24 59	—	56	68	++	—
54	16	18	00	00	00	2,3,6-Trihydroxybenzoic acid	—	07 60	23	21	08	62	—	03	50 69	+	+
83	01	78	77	35	08	3,5-Dimethoxy-4-hydroxybenzoic acid	+	08 15	21	35 20	15 35	33	—	—	68	++	+
89	08	88	90	79	69	3,4,5-Trimethoxycinnamic acid	32	—	—	—	—	—	—	07	—	++	+

TABLE 6

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME NAPHTHALENE AND QUINOLINE DERIVATIVES

R_F values × 100						Compounds	Detection									
F	E	A	B	C	D		U.V.	D1	D2	D3	D4	DB	DN	Fe	Mo	Mn	Ind
93	93	93	90	86	86	4-Chloro-1-naphthol	30	14 21	23	25	15 61	35 28	—	24	25 28	++	—
55	93	00	00	00	00	1-Amino-4-naphthol	69	23	61	61	24	68	59	56	68	++	—
91	93	92	93	10	03	2-Methyl-4-amino-1-naphthol	71	23	+	+	23	—	—	05	50 30	++	—
82	87	87	59	27	04	1-Amino-7-naphthol	33	22 65	25	28	28 23	23	—	69	24 69	+	—
77	85	75	56	21	04	2-Amino-7-naphthol	69	10	19	23	19 24	68	60	56	68	++	—
88	87	85	82	62	11	2-Amino-3-naphthol	30	10 64	23	24	19 68	42	—	+	68	+	—
94	57	94	92	92	75	2-Hydroxy-1-naphthoic acid	34	11	23 65	24 25	12	45	17	69	69	++	++
92	56	93	84	84	54	3-Hydroxy-2-naphthoic acid	03	12 15	25	25 28	15	45	—	28 68	71	++	++
93	85	93	92	93	94	5-Hydroxy-1,4-naphthoquinone = juglone	56	—	—	—	—	—	+	+	—	+	—
91	79	92	74	68	19	2,3-Dihydroxynaphthalene	—	62 63	23 25	25 68	19	51	—	29	68	++	—
91	05	87	92	90	92	5,8-Dihydroxy-1,4-naphthoquinone = naphthazarin	56	71	69	24	03	26	17	71	71	—	—
83	76	69	82	58	52	2-Hydroxyquinoline	30	—	—	—	—	—	—	—	—	+	—
71	58	33	45	03	00	4-Hydroxyquinoline	34	—	—	—	—	—	—	13	—	—	—
58	76	54	69	09	01	5-Hydroxyquinoline	69	08 14	65 23	65 25	10	35	01	51	07 69	++	—
73	89	78	90	68	67	8-Hydroxyquinoline	57	14	15	23	63	35	—	39	69	+	—
68	55	35	69	03	00	4-Hydroxyquinaldine	—	—	—	—	—	—	—	08	—	—	—
57	92	62	93	56	47	8-Hydroxyquinaldine	—	12 21	15	65 63	12 61	30 41	—	70	68	+	—
74	02	49	dec.*	00	00	2,4-Dihydroxyquinoline	34	06	19	23	08	69	—	—	—	+	—
59	00	15	00	00	00	4,8-Dihydroxyquinoline-2-carboxylic acid = xanthurenic acid	34	17	—	—	17	40	—	—	—	—	+
51	28	16	17	00	00	2,3-Dihydroxyquinoxaline	32	—	—	—	—	—	—	—	—	—	—

* Decomposition.

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TABLE 7

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME BENZOIC AND PHTHALIC ACID DERIVATIVES

R_F values × 100						Compounds	Detection									
F	E	A	B	C	D		U.V.	D1	D2	D3	D4	DB	DN	Fe	Mo	Mn	Ind
90	33	91	89	79	39	Benzoylformic acid	34	08	11	63	09	38	06	—	71	+	+
93	93	92	89	90	90	*p*-Chlorobenzaldehyde	—	—	—	—	—	—	06	—	—	+	—
91	39	87	88	87	83	2,4-Dichlorobenzoic acid	—	—	—	+	—	—	—	—	—	—	++
93	53	95	88	88	72	3,5-Dichloroanthranilic acid	38	—	—	—	—	—	—	—	—	+	+
92	05	88	49	37	06	2-Chloro-4-aminobenzoic acid	—	—	—	+	07	—	—	57	—	+	+
90	19	89	89	82	33	2-Acetamidobenzoic acid	30	—	—	—	—	—	—	04	—	—	+
86	03	83	43	09	01	4-Acetamidobenzoic acid	—	—	—	—	—	—	—	—	—	—	+
32	02	02	00	00	00	2,5-Diaminobenzoic acid	71	—	60	—	59	26 63	—	55	44 7[c]	++	+
41	00	11	02	00	00	3,5-Diaminobenzoic acid	38	12	65	23	15	39 23	—	59	—	++	+
92	48	89	81	84	48	3,5-Dinitrobenzoic acid	65	—	—	—	—	—	—	—	—	—	+
90	12	92	84	69	28	*o*-Carboxybenzaldehyde	—	—	—	—	—	—	01	—	—	+	+
94	93	92	96	95	95	Terephthalaldehyde	—	—	—	—	—	—	06	—	—	+	—
92	00	87	64	35	00	5-Methylisophthalic acid = uvitic acid	—	—	—	—	—	—	—	—	—	—	+
86	00	68	05	00	00	5-Aminoisophthalic acid	38	—	+	+	06	—	—	58	—	+	+
92	00	92	38	29	00	5-Nitroisophthalic acid	34	—	—	—	—	—	—	—	—	—	+
88	00	79	31	17	00	2-Nitroterephthalic acid	71	—	—	—	—	—	—	—	—	—	++
92	02	89	80	59	00	Diphenic acid = diphenyl-2,2′-dicarboxylic acid	—	+	—	+	—	—	—	—	—	—	++
57	00	02	00	00	00	Prehnitic acid	—	—	—	—	—	—	—	—	—	—	++

In that case the compounds do not react with the diazonium reagents and dibromoquinonechloroimide.

In general, the R_F values seem to follow the same pattern as found earlier, although the compounds given in the present tables are more heterogeneous, and should be regarded together with the values obtained before. The introduction of an amino group gives a maximum value for solvent E, the R_F values in all other solvents being lowered considerably, even down to 00 in solvents C and D. The presence of a free carboxyl group is demonstrated by a low value in solvent E and this is significant. This holds also for monohydric phenols. It is interesting to note the behaviour of the monohydroxypyridines (Table 1) in the different solvent systems. The second maximum value in solvent B, besides that in F or E, was not found before for any of the types of compounds investigated by this procedure. Hydroxyquinolines and hydroxyquinaldines (Table 6) behave similarly. Also all the alkaloids in Table 13 show the same type of R_F pattern in these solvents.

The limited space allows no detailed discussion here. It is hoped to present the condensed results of the two paper-chromatographic studies together later on.

Table 9 gives data for pyridinedicarboxylic acids; no colour reactions and zero values for R_F in solvents E, C and D remind one of the values obtained for the three phthalic acids. In Tables 10 to 14 many compounds of metabolic origin are found. The derivatives of aromatic amino acids are only practically separated in solvent F and this could therefore be regarded as significant for this type of compound. When

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TABLE 8

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME AROMATIC NON-PHENOLIC AND HETEROCYCLIC MONOCARBOXYLIC ACIDS WITH THE COOH GROUP IN THE SIDE CHAIN, AND THEIR DERIVATIVES

R_F values × 100						Compounds	Detection									
F	E	A	B	C	D		U.V.	D1	D2	D3	D4	DB	DN	Fe	Mo	Mn	Ind
90	29	87	92	89	88	α-Methylcinnamic acid	—	—	—	—	+	—	—	03	71	++	+
92	22	90	90	89	83	2-Chlorocinnamic acid	—	—	—	+	—	—	—	—	—	+	+
92	18	90	91	88	86	3-Chlorocinnamic acid	—	—	—	+	—	—	—	—	—	+	+
94	20	95	90	88	65	3-Nitrocinnamic acid	65	—	—	—	—	—	—	—	—	+	+
90	28	88	67	61	12	*p*-Chloromandelic acid	—	—	—	—	—	—	—	04	—	+	+
86	08	81	72	36	06	*dl*-Tropic acid	—	—	—	—	—	—	—	—	—	+	+
87	28	82	78	67	22	*dl*-Atrolactic acid	—	—	—	—	—	—	—	—	—	+	+
94	33	92	92	92	91	2-Phenyl-*n*-butyric acid	—	—	—	—	—	—	—	—	—	—	+
93	48	89	89	89	87	Diphenylacetic acid	—	—	—	+	—	—	—	—	+	—	+
49	00	27	34	02	00	*p*-Aminophenylacetic acid	48	59	—	10	07	—	—	—	+	+	+
92	92	85	92	91	92	*p*-Nitrophenylacetic acid	71	—	—	—	—	03, 40	—	—	—	—	+
87	07	83	71	54	18	2-Furoic acid	34	—	—	—	—	—	—	03	—	+	+
89	13	88	89	83	74	β-(2-Furyl)-acrylic acid	57	—	56	25	07	—	—	60	71	++	+
92	19	86	82	79	62	2-Thiophenecarboxylic acid	—	—	—	—	—	—	—	03	—	+	+
11	09	00	00	00	00	4-Imidazolecarboxylic acid	34	08	—	63	07	—	02	—	—	+	+
24	01	02	01	00	00	Imidazolyl-4 (or 5)-acrylic acid = urocanic acid	—	07	17, 58	20, 65	09	—	—	+	—	++	+
08	21	05	04	00	00	Imidazolylpyruvic acid	33	61	60	23	60	—	+	51	69	+	+
90	88	93	89	79	50	3-Indolealdehyde	34	—	—	-	—	—	08	—	71	+	—
84	05	82	42	18	02	*dl*-2-(3-Indolyl)-lactic acid	—	—	—	—	—	—	—	—	—	+	+
46	05	16	28	00	00	Isonicotinic acid	—	—	—	—	—	—	—	—	—	—	+
29	02	09	47	03	01	6-Methylpicolinic acid	—	—	—	—	—	—	—	—	—	—	+
95	07	64	82	00	00	6-Quinolinecarboxylic acid	—	—	—	—	—	—	—	—	—	—	+

TABLE 9

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF PYRIDINEDICARBOXYLIC ACIDS

R_F values × 100						Compounds	Detection* Ind
F	E	A	B	C	D		
45	00	03	00	00	00	2,3-Pyridinedicarboxylic acid = quinolinic acid	+
58	00	11	00	00	00	2,4-Pyridinedicarboxylic acid = lutidinic acid	+
54	00	15	09	00	00	2,5-Pyridinedicarboxylic acid = isocinchomeronic acid	+
96	00	41	33	06	00	2,6-Pyridinedicarboxylic acid = dipicolinic acid	+
40	00	02	00	00	00	3,4-Pyridinedicarboxylic acid = cinchomeronic acid	+
77	00	43	13	00	00	3,5-Pyridinedicarboxylic acid = dinicotinic acid	+

* The compounds listed in this table gave no reaction with the reagents used for detection in the previous tables, except Ind.

the amino group is acetylated the compounds behave in general like carboxylic acids. One example of this is acetyltryptophan, and more examples are to be found in Table 14, where the hippuric acids are tabulated. Although the structure of these compounds is rather similar, the only differences being the presence of other substituents in the aromatic part of the molecule, chromatographic separation is limited to three solvents, A, B and C. The difficulty in distinguishing between several struc-

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TABLE 10

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME AROMATIC, HETEROCYCLIC AND ALIPHATIC AMINO ACID DERIVATIVES

R_F values × 100						Compounds	Detection									
F	E	A	B	C	D		U.V.	D_1	D_2	D_3	D_4	DB	NH*	Fe	Mo	Mn	Ind
42	07	02	02	00	00	DL-*p*-Chlorophenylalanine	—	—	—	—	—	—	23	—	71	+	—
30	04	01	00	00	00	*o*-Fluorophenylalanine	—	—	—	—	06	+	23	—	—	—	—
29	05	01	00	00	00	*m*-Fluorophenylalanine	—	—	—	—	07	+	23	—	—	—	—
31	05	01	00	00	00	*p*-Fluorophenylalanine	—	—	—	—	06	—	23	—	—	—	—
44	04	02	00	00	00	DL-Thyronine	—	14	—	63	09	35	23	—	68	++	—
00	05	00	00	00	00	DL-Thyroxine	—	06	—	—	62	—	58	—	—	+	—
00	00	00	00	00	00	3-Aminotyrosine	34	10, 59	62	56	11, 56	59	23	23	68	++	—
83	03	81	53	11	01	DL-Acetyltryptophan	—	—	—	—	07	+	—	—	—	++	+
35	03	01	00	00	00	DL-4-Methyltryptophan	—	—	—	+	08	22	23	—	71	+	—
27	00	00	00	00	00	DL-4-Hydroxytryptophan	34	—	+	24	—	26, 71	23	—	71	+	—
37	03	00	00	00	00	DL-5-Methyltryptophan	—	—	—	59	07	—	23	—	—	+	—
13	01	00	00	00	00	DL-5-Hydroxytryptophan	—	19, 62	65	25	63	35	+	+	68	++	—
27	02	00	00	00	00	Kynurenine	40	—	—	—	—	—	23	—	—	+	—
22	00	00	00	00	00	3-Hydroxykynurenine	48	14	23, 65	63	14, 24	41	59	62	51, 42	+	—
00	00	00	00	00	00	Histidine	34	13	11	15, 62	07	—	24	—	—	+	—
00	02	00	00	00	00	DL-1-Methylhistidine	71	+	57	60	07	—	23	—	—	+	—
04	28	00	00	00	00	6-Aminonicotinamide	—	—	—	—	06	—	—	—	06, 72	+	—
48	02	11	01	00	00	6-Hydroxynicotinic acid	—	—	—	—	—	—	—	—	58	+	+
10	01	00	00	00	00	DL-Pipecolic acid	—	—	—	10	06	—	26	—	—	—	+
17	01	00	00	00	00	α-Aminophenylacetic acid	—	—	—	—	—	—	—	—	—	—	+
90	00	91	52	26	00	N-*o*-Carboxyphenyl-glycine	41	07	—	08	10	41	—	52	71	++	+
87	81	04	05	00	00	N-*p*-Hydroxyphenyl-glycine	—	—	—	+	—	—	—	—	+	—	+
06	00	00	00	00	00	DL-2-(3,4-Dihydroxyphenyl)-glycine	—	17	19	17	07	51, 71	—	42	68	+	—
73	01	33	03	00	00	*p*-Aminobenzoyl-L-glutamic acid	57	—	—	+	06	—	58	—	+	++	++
84	09	80	86	42	10	ω-Benzoylamino-*n*-valeric acid	—	—	—	—	—	—	—	—	—	—	+
85	11	83	88	60	18	ω-Benzoylamino-*n*-caproic acid	—	—	—	—	—	—	—	—	—	—	+
47	00	07	05	00	00	Formylglycine	34	—	—	—	—	—	24	—	—	—	++
02	00	00	00	00	00	Sarcosine	—	—	—	08, 72	06	59	24	57	—	+	—
54	00	08	14	00	00	Aceturic acid	—	—	—	—	—	—	23	—	—	—	++
35	00	01	00	00	00	Ureidosuccinic acid	—	—	—	—	—	—	26	—	—	—	+

* NH = ninhydrin reagent.
DN reagent gave no reaction with the compounds listed in this table.

tures is somewhat lessened by the use of several reagents for detecting hippuric acids, as pointed out in the section on additional reagents. Although the shades produced by these reagents are rather similar, the time within which they develop is in some cases decisive.

The main characteristic of biologically active nitrogen compounds of amine type is their high R_F value in E, and also values other than 0 in F. For indole derivatives no generalization can be made, since it depends on the substituents to which type they belong; in the case of OH- substituents, or only alkyl residues, they belong

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TABLE II

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME BIOLOGICALLY ACTIVE NITROGEN COMPOUNDS

R_F values × 100						Compounds	Detection										
F	E	A	B	C	D		U.V.	D1	D2	D3	D4	DB	DN	Fe	Mo	Mn	Ind
41	85	02	04	00	00	β-Phenylethylamine	—	—	—	08	06	—	—	—	—	—	—
19	88	00	00	00	00	*dl*-β-Hydroxyphenylethylamine	—	—	—	59	—	—	—	—	—	+	—
39	88	02	12	00	00	*d*-Amphetamine	—	—	—	+	—	—	—	—	—	—	—
17	dec.*	00	00	00	00	3-Hydroxythyramine = DOPA-amine	—	15	20	21	60	25	—	45	68	++	—
18	26	00	00	00	00	3,4-Dihydroxynorephedrine = corbasil	—	17	17	17	65	26	—	51	69	+	—
40	85	00	09	00	00	Hordenine	—	13	60	62	07	26|64	—	—	—	++	—
08	02	00	02	00	00	Mescaline	—	—	—	—	—	—	—	—	—	+	—
01	28	00	00	00	00	Histamine	—	09	—	64|10	64	—	—	—	—	—	—
05	00	00	00	00	00	Trigonelline	—	—	—	—	—	—	—	57	—	—	—
95	92	96	96	94	95	2-Methylindole	03	07	+	62	11	24	—	—	+	+	—
94	96	93	96	93	93	5-Methylindole	—	—	—	—	09	24	—	—	71	+	—
44	87	03	00	00	00	Tryptamine	—	—	—	08	07	23	—	—	—	++	—
36	83	01	00	00	00	Bufotenine	—	17	65|23	23	62	37	—	—	69	+	—
28	12	07	12	00	00	Adrenochrome	57	—	—	—	—	70	—	—	71	+	—
71	88	07	64	01	00	Lysergic acid diethylamide tartrate	33	—	—	—	—	—	—	—	—	—	—
27	32	01	02	00	00	4-Desoxypyridoxine	—	11	+	25	12|19	35	—	62	71	+	—
02	53	00	00	00	00	Pyridoxamine	33	08|11	60|65	63|24	12	38|35	—	62	71	+	—
28	23	02	02	00	00	4-Pyridoxic acid	32	08	+	23	09	38	—	58	—	+	+
02	00	00	00	00	00	Pyridoxal-5-phosphate	34	—	—	+	10	39	01	—	—	—	+
18	03	00	01	00	00	Creatinine	—	—	—	—	—	—	—	58	+	—	—
09	00	00	00	00	00	Riboflavine-5′-phosphate	03	—	—	—	—	—	—	—	—	+	++
30	00	02	01	00	00	Hydantoic acid**	—	—	—	—	—	—	—	—	—	—	+
33	23	05	06	00	00	Hydantoin**	—	—	—	—	—	—	—	—	—	—	—
93	91	97	97	93	92	Alloxan	—	—	—	—	—	—	—	—	—	—	33
27	11	02	00	00	00	Allantoic acid**	—	—	—	—	—	—	—	—	—	—	+
12	02	00	00	00	00	Allantoin**	—	—	—	—	—	—	—	—	—	—	—
94	03	00	00	00	00	Dialuric acid = 5-hydroxybarbituric acid	—	—	—	—	—	—	—	—	30	—	+
78	04	00	00	00	00	Parabanic acid	—	—	—	—	—	—	—	—	—	—	+
02	00	00	00	00	00	Betaine**	—	—	—	—	—	—	—	—	—	—	—
25	09	01	00	00	00	Urea**	—	—	—	—	—	—	—	—	—	—	—
03	00	00	00	00	00	Aneurine	—	—	60	60	—	—	—	—	71	+	—

* Decomposition. ** These compounds were detected by the EH and DAC reagents.

to the phenol type, giving violet-red colours with dibromoquinonechloroimide reagent. The presence of a carboxyl group gives them the character of an aromatic carboxylic acid with a side chain, and they can only be distinguished from the latter by the positive reaction with the Ehrlich reagent. This reagent is by no means selective in the same sense as the hippuric acid reagent is for hippuric acids. Some amino acid and carbamoyl derivatives produce yellow to orange colours, as has been shown by the examples given in the section on additional reagents. Also some pyrrolecarboxylic acids, urea, kynurenine and anthranilic acid derivatives give strong colours and this

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TABLE 12

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME INDOLE DERIVATIVES

R_F values × 100						Compounds	Detection													
F	E	A	B	C	D		EH	U.V. EH	DAC	U.V. DAC	U.V.	D1	D2	D3	D4	DB	DN	Fe	Mo	Mn	Ind
92	90	94	93	94	92	Indole	24	—	28 33	—	—	06	—	—	10	25	15	—	03	+	—
95	92	96	96	94	95	2-Methylindole	22	65	35 25	23	03	07	+	62	11	24	—	—	+	+	—
94	96	93	96	93	93	5-Methylindole	22	—	23 28	45	—	—	—	—	09	24	—	—	71	+	—
95	92	90	92	92	92	Indoxyl acetate	57	—	23	23	38	+	—	—	60	+	—	—	71	+	—
93	93	96	96	95	95	5-Methoxyindole	24 20	—	38 28	—	71	03	+	+	10 62	24	—	—	51 69	+	—
94	95	94	96	96	96	5-Benzyloxyindole	18 22	—	40 25	—	—	—	—	—	09	23	—	—	71	+	—
92	00	91	84	67	28	Isatin	—	—	62	—	65	07	08	60	—	—	03	—	—	+	—
90	88	93	89	79	50	3-Indolealdehyde	26	—	22 63	—	34	—	—	—	—	—	08	—	71	+	—
90	87	84	86	79	49	5-Methoxy-3-indole-aldehyde	26	—	26	—	34	—	—	—	—	—	08	—	71	+	—
87	14	90	73	65	23	α-(3-Indolyl)-acetic acid	60 70	—	22	—	—	—	—	03	07	58 17	—	—	—	+ +	+
92	15	92	83	72	42	β-(3-Indolyl)-propionic acid	23 71	—	23	—	—	—	—	—	08	—	—	—	—	+	+
94	17	93	88	83	50	γ-(3-Indolyl)-butyric acid	23 70	—	23	—	—	—	—	—	08	—	—	—	—	+	+
84	05	82	42	18	02	DL-2-(3-Indolyl)-lactic acid	26 71	—	25	64	—	—	—	—	—	—	—	—	—	+	+
75	00	49	21	03	00	DL-2-(3-Indolyl)-lactic acid, double spot	26	—	22	—	—	—	—	—	—	—	—	—	—	+	+

(continued on p. 107)

TABLE 12 (*continued*)

R_F values × 100						Compounds	Detection													
F	*E*	*A*	*B*	*C*	*D*		*EH*	*U.V. EH*	*DAC*	*U.V. DAC*	*U.V.*	*D1*	*D2*	*D3*	*D4*	*DB*	*DN*	*Fe*	*Mo*	*Mn*	*Ind*
88	08	93	80	58	12	5-Methoxy-2-carboxy-indole	26 25	—	70	—	33	—	25	24	+	—	—	59	—	—	+
92	03	80	07	04	00	5-Hydroxy-3-indoleacetic acid	68	—	24	68	03	14	65	25	62	68	—	—	30	++	+
36	03	00	00	00	00	L-Tryptophan	57	—	23	—	—	—	—	—	06	—	—	—	—	+	—
83	03	81	53	11	01	DL-Acetyltryptophan	60 26	63	25 22	20	—	—	—	—	07	+	—	—	—	++	—
35	03	01	00	00	00	DL-4-Methyltryptophan	—	—	23	—	—	—	—	+	08	22	—	—	71	+	—
27	00	00	00	00	00	DL-4-Hydroxytryptophan	71	—	26 52	65	34	—	+	24	—	26 71	—	—	71	+	—
37	03	00	00	00	00	DL-5-Methyltryptophan	—	—	23 25	—	—	—	—	59	07	—	—	—	—	+	—
13	01	00	00	00	00	DL-5-Hydroxytryptophan	—	—	26	—	—	19 62	65	25	63	35	—	+	68	++	—
44	87	03	00	00	00	Tryptamine	70 51	—	23	—	—	—	—	08	07	23	—	—	—	++	—
53	84	01	09	00	01	Gramine	57	66	22	65	—	—	07	07	11	24	—	—	03	+	—
38	64	00	00	00	00	Serotonine	71	—	26	—	65	20	24	25	63	35	—	70	28	+	—
36	83	01	00	00	00	Bufotenine	27 71	70	30 25	19	—	17	65 23	23	62	37	—	—	69	+	—
28	12	07	12	00	00	Adrenochrome	—	—	—	—	57	—	—	—	—	70	—	—	71	+	—
71	88	07	64	01	00	Lysergic acid diethyl-amide tartrate	24	—	24	—	33	—	—	—	—	—	—	—	—	—	—
86	95	44	91	12	05	Reserpine*	—	—	—	—	34	—	—	—	—	—	—	—	—	—	—
57	85	02	23	00	00	Yohimbine*	—	—	—	—	34	—	—	—	—	—	—	—	—	—	—
48	92	03	42	03	03	Physostigmine*	—	—	—	—	—	—	—	09	06	—	—	—	71	+	—
32	67	02	75	03	01	Brucine*	—	—	—	—	—	—	—	—	—	—	—	—	—	+	—
43	83	02	65	03	01	Strychnine*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

* Also detected by Bi reagent.

TABLE 13

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME NATURAL PRODUCTS OF VEGETABLE AND MOULD ORIGIN

R_F values × 100						Compounds	Detection										
F	E	A	B	C	D		U.V.	D1	D2	D3	D4	DB	DN	Fe	Mo	Mn	Ind
75	72	55	89	60	55	2,6-Dimethyl-γ-pyrone	—	—	—	64	—	—	—	+	—	+	—
89	07	82	86	84	80	Coumarin-3-carboxylic acid	34	—	—	—	—	—	—	—	—	+	+
89	55	84	43	13	00	5,7-Dihydroxy-4-methylcoumarin	33	12	66	65	12	35	—	—	03 33	+	—
76	03	23	05	00	00	Quercitrin	56	07	15 63	62	58	47	—	50	08 51	++	—
84	67	58	00	00	00	*d*-Catechin	—	10	65	65	11	24	—	51	09 42	++	—
37	13	01	00	00	00	Neohesperidin	—	07	11	63	08 65	33 35	—	57 56	71	+	—
88	06	73	33	05	00	Gibberellic acid	—	—	—	—	—	—	—	—	—	+	+
13	76	00	03	00	00	Cinchonine*	34	—	—	—	—	56	—	—	—	72	—
12	77	00	00	00	00	Morphine*	—	13	58	60	57	52	—	71	30	++	—
47	91	00	14	00	00	Atropine*	—	—	—	—	—	—	—	—	—	—	—
57	85	02	23	00	00	Yohimbine*	34	—	—	—	—	—	—	—	—	+	—
86	95	44	91	12	05	Reserpine*	34	—	—	—	—	—	—	—	—	—	—
38	76	02	30	00	00	Pilocarpine*	—	—	—	—	—	—	—	—	—	—	—
48	92	03	42	03	03	Physostigmine*	—	—	—	09	06	—	—	—	71	+	—
32	67	02	75	03	01	Brucine*	—	—	—	—	—	—	—	—	—	+	—
43	83	02	65	03	01	Strychnine*	—	—	—	—	—	—	—	—	—	—	—
37	01	03	00	00	00	Isoascorbic acid	34	72	—	—	+	72	—	72	38	++	+
92	45	92	61	43	00	Alternariol	38	62	23	65	08	25	—	71	—	+	—
92	85	88	88	87	42	Alternariol monomethyl ether	38	60	+	65	—	24	—	—	—	+	—
92	06	86	92	23	06	Stipitatonic acid	48	08	63	22 65	11	56	72	58	—	+	—
04	02	00	00	00	00	Aureomycin	03	+	+	17	08	70 56	—	58	—	—	—
39	02	07	07	00	00	Terramycin	03	—	—	07 69	11	26	—	57	—	—	—
90	91	86	71	42	06	Chloromycetin = chloramphenicol	65	—	—	—	—	—	—	—	—	+	—

* Also detected with Bi reagent.

TABLE 14

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME HIPPURIC ACID DERIVATIVES

R_F values × 100						Compounds	Detection										
F	E	A	B	C	D		U.V.	D1	D2	D3	D4	DB	DAB*	Fe	Mo	Mn	Ind
86	07	70	58	12	00	Hippuric acid	—	—	—	—	—	—	08	—	—	—	+
88	14	85	82	47	04	L-Benzoylalanine	—	—	—	—	—	—	+	—	—	—	+
78	05	38	15	02	00	α-Hydroxyhippuric acid	—	—	—	—	—	—	+	—	—	—	+
92	05	86	56	32	02	*o*-Hydroxyhippuric acid	38	06	—	+	08	38	08 17	23	—	+	+
78	06	53	10	00	00	*m*-Hydroxyhippuric acid	—	07	—	+	+	38	06 17	—	—	+	+
79	04	44	04	00	00	*p*-Hydroxyhippuric acid	—	+	—	+	60	—	08	—	—	+	+
84	07	72	83	31	06	*o*-Methoxyhippuric acid	—	—	—	—	—	41	07	—	—	—	+
83	08	67	65	13	01	*p*-Methoxyhippuric acid	—	—	—	—	—	—	09	—	—	—	+
84	12	72	67	27	03	*o*-Chlorohippuric acid	—	—	—	—	—	—	08 13	—	—	—	+

(continued on p. 109)

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TABLE 14 (*continued*)

R_F values × 100						Compounds	Detection										
F	E	A	B	C	D		U.V.	D1	D2	D3	D4	DB	DAB*	Fe	Mo	Mn	Ind
88	11	84	68	30	04	*o*-Iodohippuric acid	—	—	—	—	—	—	+	—	—	—	+
69	03	24	07	00	00	*p*-Aminohippuric acid	34	—	—	63	08	—	07, 11	+	+	+	+
91	10	77	53	20	01	*m*-Nitrohippuric acid	65	—	—	—	—	—	08, 13	—	—	—	+
88	12	82	57	18	01	*p*-Nitrohippuric acid	56	—	—	—	—	—	62, 65	—	—	—	+
91	17	87	48	36	02	3,5-Dinitrohippuric acid	65	—	—	—	—	—	62	—	—	—	+
68	01	18	00	00	00	3,4-Dihydroxyhippuric acid	—	—	—	—	07	—	+	42	71	+	+
89	09	78	77	27	02	Cinnamoylglycine	—	—	—	64	—	—	08, 17	—	—	++	+
85	08	70	69	15	01	Phenaceturic acid	—	—	—	—	—	—	—	—	—	—	+
85	04	70	32	08	00	*o*-Hydroxyphenaceturic acid	—	08	11	65, 63	23, 19	33, 28	22	+	71	+	+
92	10	52	34	02	00	Phenacetylglutamic acid	—	—	—	—	—	—	—	—	—	—	+
88	02	75	80	33	03	Phenoxyaceturic acid	—	—	—	—	—	—	—	—	—	—	+
49	02	08	12	00	00	Nicotinuric acid	—	—	—	—	—	—	08, 17	—	—	—	+
43	00	03	00	00	00	Phthaloylglycine	—	—	—	—	—	—	11	—	—	—	+

* DAB = hippuric acid reagent. DN reagent gave no reaction with the compounds listed in this table.

TABLE 15

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME MISCELLANEOUS ALIPHATIC ACIDS

R_F values × 100						Compounds	Detection										
F	E	A	B	C	D		U.V.	D1	D2	D3	D4	DB	DN	Fe	Mo	Mn	Ind
27	01	02	10	00	00	Glyoxylic acid	—	—	—	—	—	—	+	—	—	+	++
70	02	37	28	04	00	β-Hydroxybutyric acid	—	—	—	—	—	—	—	—	—	+	++
76	10	31	09	00	00	β-Hydroxy-β-methylglutaric acid	—	—	—	—	—	—	—	—	—	23	+
91	16	92	93	88	88	DL-Thioctic acid	—	—	—	—	—	52	—	—	—	++	+
82	00	61	19	03	00	Thiomalic acid	—	—, 72	—, 72	—, 72	—, 72	60	—	38, 72	49, 30	++	++
40	00	18	06	00	00	Dihydroxyfumaric acid	57	72	72	09, 72	05	72	—	72	47, 30	++	++
85	00	77	61	17	00	3-Methyladipic acid	—	—	—	—	—	—	—	—	—	—	+
89	01	82	73	28	02	3,3-Dimethylglutaric acid	—	—	—	—	—	—	—	—	—	—	++
90	00	91	88	67	00	Sebacic acid	—	—	—	—	—	—	—	—	—	—	+

should be borne in mind when dealing with indole derivatives. The modified reagent, which was prepared on the basis of *p*-dimethylaminocinnamaldehyde in hydrochloric acid has some advantage over the common Ehrlich reagent, since the colours are in most cases stronger and red and blue shades dominate, which sometimes makes it possible to discern the spots more easily.

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5. SUMMARY

Supplementary data are given for the paper-chromatographic investigation of mono-, di- and trihydric phenol derivatives and various compounds of biochemical interest, such as biogenic amines, aromatic and heterocyclic amino acids, hippuric acids, indole derivatives. Some further types of compounds such as alkaloids, hydroxylated quinoline and pyridine derivatives and antibiotics, not dealt with in previous investigations reported earlier in these Reviews, have now been included. The R_F values of these compounds in six different solvent systems together with the corresponding colour reactions produced by ten standard reagents have been determined and are presented in 15 tables. Altogether approx. 270 compounds have been investigated and 20 hippuric acid derivatives synthetized. Application of these procedures to the clinical-chemical analysis of urine samples in order to detect metabolic excretion or detoxification products is indicated.

The hippuric acid reagent was modified so that it contained *p*-dimethylaminocinnamaldehyde in acetic acid anhydride. The Ehrlich reagent was prepared on the basis of *p*-dimethylaminocinnamaldehyde in hydrochloric acid; the colours produced with this reagent made it possible to differentiate the indole derivatives more distinctly.

The relation between the chemical structure of the compounds studied and the R_F values and colours produced with the detection reagents, is discussed.

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CHROMATOGRAPHY OF LIPIDS ON SILICIC ACID

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1. INTRODUCTION

Twenty years have passed since KAUFMANN[1] and TRAPPE[2] first reported chromatography of lipids on silicic acid. For ten years relatively little further work was reported[3–18], but after 1950 the number of references in the literature to this technique rose roughly exponentially, until in 1959 (when there were well over 100) the rise clearly tapered off. It seems appropriate now to summarize what has been learnt about silicic acid chromatography of tissue lipids and similar compounds that are of interest.

Development of chromatographic methods has made most problems in lipid chemistry and biochemistry more tractable than they were previously. The problem of resolving lipid extracts from natural sources on a preparative scale into separate, intact, molecular species has been investigated most successfully by column chromatography on silicic acid. Whether it could be completely solved by using silicic acid or any other adsorbent is a matter for speculation, or rather, for future investigation. The work of TRAPPE[3], BORGSTRÖM[19–21], FILLERUP AND MEAD[22, 23], and LEA, RHODES AND STOLL[24] established that a silicic acid column can separate a natural lipid mixture into two classes, "neutral lipids" and phospholipids, and can resolve these into more classes (*e.g.* triglycerides, sterols, etc.). This ability to differentiate between lipid classes, largely uninfluenced by olefinic unsaturation, accounts chiefly for the present-day popularity of silicic acid as an adsorbent. Moreover, since good silicic acid adsorbents are unreactive at room temperatures, lipids can be recovered after chromatography in yields of 100%, within limits of error in measurement. Labile compounds, such as hydroperoxides[25], allylic tertiary alcohols[26–28], and plasmalogens are safe.

The bibliography in this review is restricted by avoiding reference to papers that mention merely the use of methods described elsewhere, and in the section on "The Adsorbent" it is intentionally less than comprehensive.

2. THE ADSORBENT

Silica, silica gel, and *silicic acid* are terms used synonymously in the literature[29]. They refer to powdered solids of the general formula $SiO_2 \cdot xH_2O$ which consist of porous,

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three-dimensional siloxane (tetrahedral Si·O·Si) structures with surface silanol (Si·OH) groups. There is usually a mono- or multi-molecular layer of adsorbed water, but such "free" water can be removed reversibly by heating at about 100°. At temperatures above 170° condensation of silanol groups occurs, liberating "fixed" water, and this process of thermal degradation continues with rising temperature until, at 1100°, only SiO_2 remains. Thermal degradation is rather irreversible because it destroys pore structure, and it permanently impairs adsorbent properties[30–42].

The behaviour of a sample of silicic acid as a chromatographic adsorbent depends on its content of silanol groups, free water, and chemical impurities, and on topography (including pore size and shape) and particle size.

The fundamental adsorbent property of silicic acid is attributed to the silanol groups, which in absence of adsorptive (*e.g.* free water) are free of hydrogen bonding[43, 44]. Affinity for an adsorptive is governed *primarily* by the strength of hydrogen bonding possible with it[34, 35, 44–49]. A sample of silicic acid is fully activated as an adsorbent if devoid of free water but not thermally degraded. It is progressively deactivated by water, remarkably large quantities of which can be added before the powder becomes visibly wet. Occasionally, chromatographic resolution is improved by adding free water to the adsorbent and using a less polar eluent[50, 51]. Using non-hydroxylic eluents on silicic acids containing 0 to *ca.* 17% free water the process governing chromatography is adsorption at a solid surface, whereas on silicic acids containing larger amounts of free water it is liquid–liquid partition. (This review is concerned primarily with adsorption chromatography.)

For use as an adsorbent silicic acid should be as pure as possible, since traces of impurity may appear in eluted fractions or may catalyze formation of artefacts. Walling[52] has shown that carefully prepared silicic acids have neutral surfaces, but others have acidic surfaces due to sulphuric acid remaining after preparation.

Silicic acid prepared from alkali silicates has a porous structure which is vitally important in chromatography. Studies on hydrocarbons illustrate that considerations of topography far outweigh those of gross surface area in determining capacity and selectivity for an adsorptive[53, 54]. Fine grinding of the adsorbent improves chromatographic resolution[55], but since it is tedious to prepare extremely fine powders, and since such powders form columns that are impervious to eluents, a compromise has to be made.

The above considerations show why different preparations of silicic acid may have widely different adsorbent properties, a fact that has troubled many workers. For chromatography of tissue lipids two or three American commercial preparations labelled *silicic acid* have proved far superior to others, particularly to others labelled *silica gel*. (This is the apparently fortuitous reason why most workers on lipids prefer the term *silicic acid chromatography* to the term *silica gel chromatography*. Objections can be raised to the use of either term.)

Lovern and co-workers[56–59] have reported a remarkable contrast between the behaviour of phospholipids on columns of *silicic acid* (Mallinckrodt) and *silica gel* (B.D.H.). Whereas phospholipids are eluted after neutral lipids from *silicic acid*,

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they are eluted first from *silica gel, being unadsorbed from ligroin* solution.* RILEY AND NUNN[60] have reported similarly, except that urea as well as phospholipids occurred in their ligroin eluate from *silica gel* and some phospholipids were found eluted after neutral lipids (see also LOVERN *et al.*[57]). BLOCH, LEDERER, and co-workers[61–65] have reported that "cord factor" (trehalose 6,6'-dimycolate) and related polyhydroxy lipids are strongly adsorbed from ligroin solution on *silicic acid* (Mallinckrodt) but are largely unadsorbed on *silica gel* (Davison). The small amounts of these lipids that are adsorbed on *silica gel* can be eluted by solvent mixtures that would elute them from *silicic acid.* These observations can all be explained as follows. Phospholipids and other lipids containing polar groups exist in ligroin solution in micelles with hydrocarbon surfaces, and the micelles are broken up readily by *silicic acid* but with difficulty by *silica gel.* Intact micelles are eluted as if they were hydrocarbon macromolecules. Non-lipids, such as urea, may be included in the micelles (the well-known "solubilizing" effect).

Comparing adsorption on samples of *silica gel* (Davison) and *silicic acid* (Mallinckrodt), CINES AND RUEHLEN[66] obtained Type I isotherms for the former and Type II for the latter: whereas *silica gel* showed only monolayer adsorption of 2,4-dimethylpentane and benzene vapours, *silicic acid* showed adsorption beyond the monolayer, although selectivity for adsorptive resided mainly in the monolayer. The relatively high silanol content of the *silicic acid* (fixed water = 4.75%[48]), sulphate impurity in the *silica gel,* topography (*e.g.* pore size distribution[66]), or a combination of these factors may account for the striking difference. In any case, it seems that whatever causes *silicic acid* to adsorb more than a monolayer of hydrocarbon may also allow it to break up micelles of polar lipids.

Lipids that do not contain highly polar groups (neutral lipids) are eluted in the same order—and by approximately the same solvent mixtures—from *silica gels* as from *silicic acids.*

For some purposes commercial silicic acid preparations may be used as received, but to obtain best and most reproducible results they should be pre-treated. HIRSCH AND AHRENS[67] have published a very valuable paper in which is described a method of pre-treatment that yields adsorbent of standard, high quality. Its particle diameter is small ($\lesssim 40\ \mu$), it does not unduly restrict the flow rate of eluent, and it is substantially devoid of free water and chemical impurities. HIRSCH AND AHRENS recommend that columns of this adsorbent should be washed with acetone–ether (1:1**) and ether before use. The acetone–ether removes free water, and the ether removes acetone. Removal of free water by means of dehydrating washes has often been advocated, and non-hydroxylic solvents, such as pure acetone or dioxan, apparently can be displaced by the first eluent (*e.g.* ether) before chromatography begins. Methanol and ethanol have been used in dehydrating washes, often on the mistaken assumption that they, too, can be similarly displaced. But at least a monolayer of alcohol is tena-

* For purposes of this review, ligroin = light petroleum = petroleum ether = benzine.

** Eluent compositions are given by volume. In the particular case of chloroform–methanol mixtures, volumes of the components are roughly additive: thus, for example, *25% methanol in chloroform* and *chloroform–methanol (3:1)* describe essentially the same eluent.

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ciously retained, and this affects the adsorption of lipids[48,68,69]. It seems that if methanol washes are employed some phospholipids are prematurely eluted by ether[70–72].

At 100° silicic acid is quite reactive, since siloxane oxygen, as well as silanol oxygen, exchanges[33] with the oxygen of $H_2{}^{18}O$. For practical purposes heating at 100–120° removes all volatile compounds, but in certain circumstances this might not yield a fully active adsorbent since some silanol groups condense with alcohols under mild conditions[73–75]. Deliberate modification of silicic acid, by alkylation, arylation, acylation, or siliconization, might actually provide useful new adsorbents[41,74,76,77]. Another kind of modification might be useful for specific separations or for studies of configuration: a silicic acid precipitated in presence of a chosen compound may possess specificity for that compound and for others with similar structural features[35,78,79].

Admixture of filter aids with silicic acid adsorbents, although frequently mentioned in the literature, should be avoided. As HIRSCH AND AHRENS[67] have pointed out, they introduce an unwanted variable in adsorbent quality, and adequate elution rates are possible without them.

Although pure silicic acid has a neutral surface[52], it has greater affinity for amino groups than is attributable to hydrogen bonding alone[49,80]. It also has limited cation exchange properties which are troublesome in chromatography of acidic phospholipids (see below)[80–85].

3. ELUENTS

Methanol > ethanol > 1-propanol > acetone > methyl acetate > ethyl acetate > ether > dichloromethane > benzene > toluene > 1,1-dichloroethane > 1,1,2,2-tetrachloroethane > chloroform > trichloroethylene > carbon tetrachloride > cyclohexane > ligroin.

Above is an expanded and slightly modified version of TRAPPE'S "eluotropic series", listing common solvents in order of their eluting power[2,86,87]. The order varies slightly according to the adsorbent sample and the adsorptive studied. The solvents most commonly used are methanol, ether, benzene, chloroform, and ligroin, though there seems no reason why others, including dioxan and 2-methoxyethanol, should not prove useful.

The literature provides some information on the relative merits of different eluents for particular separations. But it may sometimes be worthwhile to compare adsorption values of purified lipids from several solvent mixtures before selecting a mixture as an eluent[2,13,67]. Alternatively, eluents can be compared by using them in chromatography on silicic acid impregnated glass fibre paper (see Table 1)[88–94]. This is a better model of column chromatography than chromatography on impregnated filter paper or chromatoplaques, which consist of polysaccharides besides silicic acid. It is not a perfect model, however, because frontal analysis of the eluent is possible. Table 1 illustrates that the order of elution of lipids may vary with the eluent used: for example, in isoöctane–ether vitamin A palmitate moves more slowly than hexadecanol, but in cyclohexane–methanol it moves faster.

For chromatography of natural lipid mixtures some form of gradient elution is

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TABLE 1

R_F VALUES OF LIPIDS ON SILICIC ACID-IMPREGNATED GLASS FIBRE PAPER

Reference	*92*	*92*	*89*	*92*	*90*	*91*
Solvent	*A*	*B*	*C*	*D*	*E*	*F*
Squalene	0.92	0.94		0.90	1	
Hexadecyl palmitate	0.61	0.93		0.91	1	
Vitamin A palmitate	0	0.46		0.87	1	
Cholesteryl palmitate	0.63	0.90	0.93	0.93	1	
Cholesteryl acetate			0.96		1	
α-Tocopheryl acetate	0.32	0.83		0.91	1	
α-Tocopherol	0.42	0.79		0.91	1	
Tripalmitin			0.93		1	
Triglycerides (unsaturated)	0	0.65	0.89	0.89	1	
Hexadecanol	0.16	0.65		0.64	1	
Vitamin D_3	0.07	0.55		0.74	1	
Vitamin A alcohol	0	0		0	1	
Stearic acid, linoleic acid					1	0.95
Cholesterol	0.08	0.44	0.67	0.69	1	
7-Dehydrocholesterol	0.04	0 0.38		0.48	1	
1,3-Dipalmitin			0.53		1	
1,3-Diolein			0.48		1	
1-Monopalmitin	0		0.11		1	
1-Monoölein	0		0.11		1	
Phosphatidylethanolamines	0	0	0		0.94	0.85
Lecithins (from egg)	0	0	0	0		0.66
Lecithin (dipalmitoleyl-)	0	0	0	0	0.50	
Lecithin (distearyl-)	0	0	0	0	0.46	
Lecithin (dimyristyl-)	0	0	0	0		0.65
Sphingomyelins	0	0	0	0	0.40	0.50
Lysolecithins	0	0	0	0		0.31
Lysolecithin (2-palmitoleyl-)	0	0	0	0	0.23	
Lysolecithin (2-stearyl)	0	0	0	0	0.18	

R_F values differ slightly on different batches of impregnated paper. Mixtures of compounds with close R_F values (*e.g.* the lysolecithins) have not been resolved.

Solvents. A: cyclohexane. B: 2% ether in isoöctane. C: 5% ether in isoöctane. D: 1% methanol in cyclohexane. E: 50% methanol in ether. F: phenol (200 g) + ether (400 ml) + acetone (250 ml) + water (50 ml).

usually employed, namely, continuous or discontinuous, convex, linear, or concave gradient elution (Fig. 1). Most workers have hitherto used discontinuous concentration gradients, in spite of their tendency to give factitious peaks in elution curves. Columns require less attention when continuous concentration gradients are used. Moreover, the symmetry and resolution of peaks is improved *if a suitable gradient is chosen*. A continuous convex gradient of methanol in chloroform resolves cerebrosides adequately[95,96] but phospholipids very poorly[97]; the latter are resolved well by a continuous concave gradient[98]. For resolution of as many components of a natural

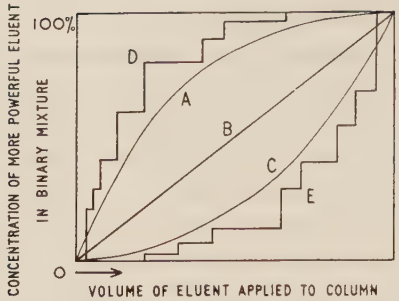

Fig. 1. Concentration gradients: continuous convex (A), linear (B), and concave (C); discontinuous convex (D) and concave (E).

lipid mixture as possible two or more successive gradient elutions are necessary, *e.g.* ligroin → chloroform → methanol or ligroin → ether → ethanol → methanol.

Eluents should be of analytical reagent grade or purer. Chloroform when purchased contains, as a preservative, 0.75–2% ethanol which gradually saturates a silicic acid column (the R_F value of ethanol in chloroform is low[99]). The effect on elution behaviour is remarkably slight, but sometimes it is worthwhile to remove the preservative just before use.

4. EXPERIMENTAL CONSIDERATIONS

For general information on techniques see LEDERER AND LEDERER[29], KORN[100], NEHER[101], and HIRSCH AND AHRENS[67].

Lipids must be chromatographed with considerable care, to avoid degradation and contamination as far as possible. Natural lipid mixtures are particularly demanding, since they require long elution periods (*e.g.* 200 hours) and large volumes of eluents (*e.g.* 150 ml per gram of adsorbent). HIRSCH AND AHRENS[67] suggest that control of column temperature is important. Adsorbent should never be re-used after washing, because contamination could result, and previously used eluents such as alcohols cannot be entirely washed away.

The susceptibility of lipids, particularly tissue phospholipids, to autoxidation makes it desirable to chromatograph them anaerobically. Therefore some workers have used nitrogen or carbon dioxide atmospheres and deoxygenated adsorbents and eluents[16, 56, 57, 70, 102–110]. The adsorbents were deoxygenated thermally or by washing with hydroquinone[109] or linoleic acid[56, 57] solutions.

Overloading of columns, which vitiated some early work, must be avoided. The load of a natural lipid mixture should not exceed 50 mg per gram of adsorbent, but frequently one tenth of this load is preferred. The load of a lipid preparation that has already been fractionated should be lower.

When a particular type of lipid has to be prepared it is worthwhile to choose a source in which that type is abundant, since adsorbent, eluents, and time can thus be saved. The purity of the product can be improved by re-chromatography. For example, to prepare a lecithin sample an extract from hen's eggs, freed of non-lipid contaminants by an appropriate method, can be run on to a column in 15% methanol

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in chloroform; after prolonged elution with this mixture, 20% methanol will elute the lecithin peak, and this can be re-chromatographed.

It is better to support the adsorbent on a sintered glass plate and/or a glass wool plug than on a filter paper or a cotton wool plug, because cellulose adsorbs polar lipids from some eluents[97]. Using suitable adsorbent samples (see above) in columns with moderate height: diameter ratios (*e.g.* 5:1), the rate of flow of most eluents is satisfactory. If pressure or suction is ever applied to improve the rate of flow it should be moderate, otherwise the adsorbent packs down tighter and so defeats the purpose[97]. If the rate dwindles after prolonged elution it can sometimes be speeded by cautiously stirring the adsorbent to a depth of a few millimetres[111].

Automatic fraction collectors activated by direct volume measurement are preferable to those activated by weight, drop counting, or timing, because the density, surface tension, and rate of delivery of eluate are very considerable variables. Eluate measuring devices and delivery tubes should be designed to minimize evaporation, which causes lipid to accumulate on them.

The course of elution can be followed by one or more of numerous methods. The best single method, although laborious, is weighing the involatile portion of each fraction in a tared vessel. "Piloting" by paper chromatography, by using chromatoplaques, or by paper electrophoresis, is very widely used, as are colorimetric analyses of cholesterol, esters, aldehydes, amino compounds, and phosphorus. Infrared and ultraviolet spectrophotometry, radioactivity measurements, and hydrolytic methods are also used. It is sometimes possible actually to watch bands being eluted, as when chloroform is the eluent and the column is almost transparent.

It might be expected that the free amino acids, sugars, etc. that contaminate lipid extracts from natural sources would be retained more firmly by silicic acid than any lipid. Unfortunately this is not so[97, 111–114]. Thymine, for example, is eluted before phosphatidylethanolamines[115], and urea and glucose after, followed by numerous amino acids. In a model experiment in which solid non-lipids were placed on the top of a column in absence of lipids, WREN AND MITCHELL[111] found that 15% methanol in chloroform eluted 2-aminoöctanoic and 2-pyrrolidone-5-carboxylic acids, then glucose, cystine, leucine and proline. Higher concentrations of methanol eluted more amino acids sequentially, and only cysteine, histidine and lysine hydrochlorides were retained from pure methanol. (There was necessarily much tailing due to insolubility of these compounds in the eluents.) To avoid misleading results, non-lipid contaminants should be removed from lipid extracts before chromatography. For this purpose, selective precipitation is not effective since polar lipids form micelles around non-lipids in non-polar media. Cellulose chromatography is effective but it removes relatively polar lipids such as inositides. Liquid–liquid partition methods are also effective and are widely favoured, although they do involve losses of the most polar lipids. The FOLCH (1957) method, about which most is known[116–123], employs the system chloroform–methanol–water, and the method of BLANKENHORN AND AHRENS[124, 125] employs heptane–alcohol–water mixtures. Both methods are combined with reproducible extraction procedures, and in both partition can easily be repeated if necessary.

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Traces of adsorbent are washed during chromatography from even the most carefully prepared columns[67, 72, 95, 111, 114, 126–128]. It is not clear whether the reason for this is mechanical, or whether it is combining or dissolving effects of lipids[129] or eluents. Neither is it clear whether or not some silicon found in eluates is tissue silicon originating with the lipids[35, 129]. If eluates are evaporated and the residual lipids redissolved, silica can be removed by centrifugation or filtration[95, 126, 127]. Silica may interfere in phosphorus analysis[130], but it can conveniently be discarded after perchloric acid digestion[131].

The possibility of contamination by silicon and other elements should be borne in mind when fractions from silicic acid columns are used in biochemical systems. Lecithin fractions eluted from silicic acid are acidic enough to resist lecithinase A[97, 132].

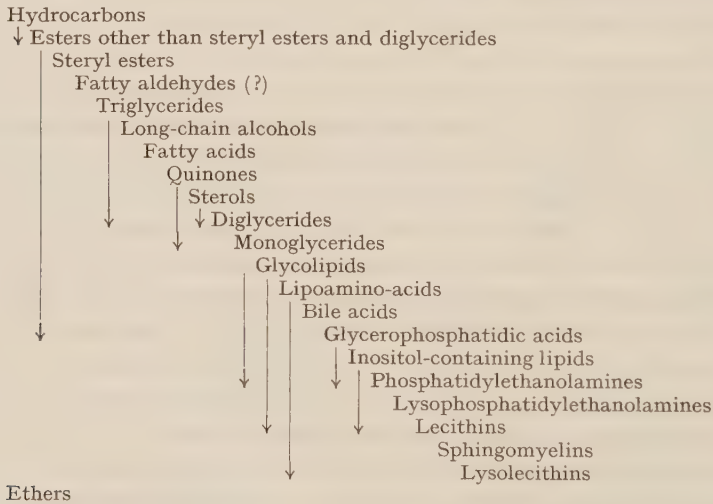
Although the emphasis of this review is on methods for preparative resolution of lipid mixtures, the use of silicic acid in biochemical microanalysis deserves mention. Micro columns (containing 0.5–1.0 g adsorbent) and impregnated papers are used widely. Phospholipids can be separated from neutral lipids by simply shaking a chloroform solution with silicic acid, which adsorbs phospholipids and can be separated by centrifugation or filtration[133–135].

5. ORDER OF ELUTION

The list in Table 2 is presented in the order of elution of the most readily eluted member of each lipid class. The arrows give some idea of the behaviour of less readily eluted members.

TABLE 2

ORDER OF ELUTION OF LIPIDS FROM SILICIC ACID



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In general, higher homologues are eluted more readily than lower homologues. Elution is retarded slightly by olefinic unsaturation, more by electronegative substituents, and most by substituents containing hydrogen atoms capable of hydrogen bonding. Further generalization is difficult, because elution behaviour is influenced by specific spatial and electronic interactions between adsorbent, eluent and adsorptive.

Each lipid class will be discussed in order.

Hydrocarbons

The saturated hydrocarbons of tissues are found in the first fraction from silicic acid, and non-polar solvents (pentane, hexane, isoöctane, ligroin, carbon disulphide) elute them before other lipids[56, 57, 67, 104, 111, 128, 136–141].

It can be assumed that hydrocarbons are eluted sequentially according to the inverse order of adsorption, acyclic alkanes > cycloalkanes > straight chain monoenes > branched chain and cyclic monoenes > dienes > benzenoid hydrocarbons and monoynes > polyenes[32, 56, 138, 139, 142–149], and under suitable conditions hydrocarbon mixtures can be fairly well resolved. SMIT[143, 144] eluted cetane before cetene with pentane, then dodecylbenzene with carbon tetrachloride. Using hexane as developing solvent on silicic acid–starch chromatostrips, KIRCHNER AND MILLER[146] found widely differing mobilities among a selection of terpenoid and other hydrocarbons. CLEMENTS[149] was able to separate *p*-menthane, α-pinene, β-pinene, and *d*-limonene (eluted in this order) on a silicic acid column at — 78°. A silica gel column 16 metres high partly resolved mixtures even such as 2,2,4-trimethylpentane–*n*-octane, *n*-dodecane–*n*-heptane, and cyclohexane–cyclopentane[142].

The polyenes, carotenes[67, 139, 150, 151] and squalene[67, 92, 138, 152], are not eluted by pure hydrocarbon solvents. HIRSCH AND AHRENS[67] found that β-carotene and squalene were eluted by 1% ether in ligroin in a fraction containing wax esters, but NICOLAIDES AND FOSTER[138] eluted squalene well before and SORRELS AND REISER[92] well after wax esters. Results of chromatography on silicic acid-impregnated glass fibre filter paper (Table 1) seem to confirm that the order of elution of these compounds is sensitive to small differences in adsorbents and eluents.

A method for quantitative determination of carotene in green leaves makes use of the fact that carotene is less strongly adsorbed than the other pigments present[153].

Esters other than steryl esters and glycerides

The naturally occurring wax esters[56, 57, 67, 92, 111, 137, 138, 141, 152] (*e.g.* hexadecyl palmitate[92, 152]) can be eluted between the hydrocarbons and steryl esters if column resolution is good. Most workers have found a slightly polar eluent necessary (*e.g.* 1% ether in ligroin[67, 92, 152]); SPENGLER AND HAUF[137] found that octadecyl stearate (also 4-hydroxystearolactone) was retained from ligroin or carbon disulphide, but eluted by carbon tetrachloride.

In 1949, RIEMENSCHNEIDER *et al.*[16] described the isolation of pure linoleic and linolenic acids by means of an elegant chromatographic experiment in which the methyl esters were fractionated on silicic acid–Hyflo Supercel (4:1, w/w) under strictly

anaerobic conditions. Further work has confirmed that fatty acid methyl[18, 67, 102, 103, 105–108, 154, 155] (and ethyl[107, 137, 156]) esters are generally eluted in order of increasing unsaturation, first by ligroin and then by low concentrations of ether[67, 102, 103, 105–108, 155] or benzene[154] in ligroin. Thus, a methyl isolinoleate has been separated from saturated and monoene esters[18], methyl arachidonate prepared from beef glands[102] and pork liver[108], and a methyl docosahexaenoate prepared from hog brain[106]. Higher homologues tend to be eluted first: for example, methyl docosapentaenoate precedes methyl eicosapentaenoate[103], and the adsorption isotherm of methyl stearate lies below that of methyl myristate[154]. Though methyl *cis*- and *trans*-oleates have not been separated quantitatively, it is interesting that the *trans*-oleate (elaidate) tends to be eluted first[105, 157]. The behaviour of methyl isanate[158] suggests that acetylenic bonds influence the order of elution only slightly. Hydroxyl substituents, on the other hand, greatly retard elution. BERGSTRÖM AND PÄÄBO[159] have found that methyl palmitate and other unsubstituted esters are eluted by pure dichloromethane, whereas methyl *threo*-9,10-dihydroxystearate and other dihydroxy esters require 0.75% methanol, and tetrahydroxy esters require 2–3% methanol in dichloromethane (which is sufficiently polar to elute some phospholipids). Bromo substituents slightly retard elution, and methyl *threo-threo*-9,10,12,13-tetrabromostearate tends to be eluted after methyl stearate[160]. R_F values recorded[94] for methyl polybromostearates on silicic acid-impregnated glass fibre paper in isoöctane are: hexa-, 0.23; tetra-, 0.47; di-, 0.62; *cf.* methyl oleate, 0.78.

p-Phenylphenacyl[161] and *p*-phenylazophenacyl[162] esters of saturated fatty acids are eluted (as fluorescent and orange bands, respectively) by ligroin–benzene mixtures in order of decreasing molecular weight. Useful separations can be obtained, *e.g.* stearate from myristate[162].

Phytyl acetate and azobenzoate[28], α-tocopheryl acetate[92], vitamin A acetate[67], and vitamin A palmitate[92, 152] are other esters of this group that have been chromatographed on silicic acid. Vitamin A acetate is somewhat difficultly eluted, and appears in the monoglyceride fraction eluted by pure ether. MARINETTI *et al.*[163] have prepared from heart muscle lipids a new keto ester, which was eluted from silicic acid by 3% ether in ligroin.

Steryl esters

Cholesteryl esters can be obtained in a well resolved fraction before the triglyceride fraction[3, 20, 22, 23, 56, 60, 67, 72, 92, 98, 104, 111, 112, 120, 127, 128, 138, 139, 152, 164–167], the preferred eluent being 1% ether in ligroin[22, 23, 60, 67, 92, 104, 139, 152, 167]. The similarity of the R_F values of cholesteryl acetate and palmitate on silicic acid-impregnated glass fibre paper (Table 1) illustrates the difficulty of column separation of steryl esters of saturated fatty acids, which has only once been reported (cholesteryl palmitate was eluted before the myristate[168]). Steryl esters of unsaturated fatty acids are more easily separated[56, 168], and KLEIN AND JANSSEN[168] have eluted cholesteryl palmitate, oleate, linoleate, and arachidonate sequentially, with 15% benzene in hexane. Apparently iodine-labelling of cholesteryl esters does not markedly affect their elution behaviour[169, 170].

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The quantitative separation of cholesteryl esters from free cholesterol that can be effected on silicic acid, together with colorimetric analysis, facilitates micro-scale study of relative amounts of these compounds in tissues (see *e.g.* refs.[164–166, 168, 171–173]). However, autoxidation in samples must be strictly avoided, since autoxidation products are eluted late[174].

Chromatographic resolution of mixtures of closely related sterols can be facilitated by prior esterification. Thus, STOKES *et al.*[175–177] separated the *p*-iodobenzoates of cholestanol, cholesterol, and Δ^7-cholestenol (decreasing order of mobility). STOKES[178] showed mobilities, dihydrolanosteryl > dihydroagnosteryl > lanosteryl > agnosteryl, for benzoates and *p*-iodobenzoates. Δ^7-Cholestenol has been isolated from skin[179] and faeces[180] as its azoate, which forms a band above that formed by cholesteryl azoate. From 3,5-dinitrobenzoylated urinary steroids KELLIE AND WADE[181] obtained the diester of pregnane-3α,20α-diol, which was eluted after the ester of cholesterol.

Fatty aldehydes

Aldehydes liberated from plasmalogens are conveniently separated from phospholipids by elution with chloroform[182, 183]. It is not known whether they are eluted as monomers or as polymers, or indeed what is their precise position in the order of elution of lipids. However, considerations of chain length and unsaturation, discussed above for esters, apply in the same way to aldehydes[184].

Triglycerides

Since the early work of TRAPPE[3] and KAUFMANN and co-workers[6–9] reports of silicic acid chromatography of triglycerides have been numerous[19–23, 56, 57, 60, 67, 72, 92, 98, 104, 111, 112, 120, 127, 128, 138, 139, 152, 156, 167, 169, 185–191]. Higher triglycerides have most often been eluted with 3–5% ether in ligroin[22, 23, 60, 67, 92, 104, 128, 139, 152, 167] or with benzene[3, 6–9, 21, 138, 185, 188, 189]. Lower triglycerides are eluted after higher[7–9, 67]. Experimenting with a selection of lipids, HIRSCH AND AHRENS[67] obtained tristearin, trilinolein, tricaproin (I, $R_1 = R_2 = R_3 = (CH_2)_4 \cdot CH_3$), and "monoacetin fat" (I, R_1 or $R_2 = CH_3$, R_2 or R_1 and R_3 long) in one fraction, "diacetin fat" (I, $R_1 = R_2$ or $R_3 = CH_3$, R_3 or R_2 long) in the next (with sterols), and tributyrin (I, $R_1 = R_2 = R_3 = (CH_2)_2 \cdot CH_3$) in the next (with dipalmitin and dilaurin); tricaproin formed a well resolved peak after tristearin. By analogy with hydrocarbons and non-glyceride esters (see

$$\begin{array}{l} CH_2 \cdot O \cdot CO \cdot R_1 \\ | \\ CH \cdot O \cdot CO \cdot R_2 \\ | \\ CH_2 \cdot O \cdot CO \cdot R_3 \end{array}$$

(I)

above) the mobility of triglycerides is generally thought to be reduced by unsaturation[56] (contrast the suggestion of MILLER AND COOPER[120]). On silicic acid-impregnated glass fibre paper the R_F value of unsaturated glycerides is 3–4% lower than that of tripalmitin (Table 1).

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Iodine-labelling in triglycerides has no appreciable effect on elution behaviour[169, 187, 191].

Long-chain alcohols

Under suitable conditions saturated, long-chain alcohols can be eluted after triglycerides[92, 152] and before free fatty acids[137, 152]. Eluents that have been used are 2% acetone in ligroin[141], 7% ether in pentane[140], 1% *tert.*-butanol in benzene[192], and chloroform (1-octadecanol is retained from carbon tetrachloride)[137]. Although resolution on their columns was incomplete, REISER and co-workers[92, 152] have demonstrated the order of elution, triglycerides > hexadecanol > vitamin A alcohol > cholesterol > vitamin D_3 (see also Table 1). KATES AND EBERHARDT[150] have found xanthophylls in chloroform eluates of green leaf lipids. DEMOLE AND LEDERER[26–28] have eluted acyclic diterpenoids with benzene in the order, phytyl acetate > isophytol (monoene, tertiary alcohol) > geranyl-linaloöl (tetraene, tertiary alcohol) > phytol (monoene, primary alcohol). This order suggests steric hindrance of adsorption of the tertiary alcohols.

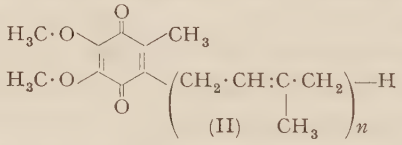
Fatty acids

The question whether free fatty acids of tissues can be separated from triglycerides on silicic acid and, if so, whether they are eluted before cholesterol, has been controversial[3, 6, 19, 20, 22, 23, 67, 104, 128, 139, 152, 167]. But in the carefully conducted experiments of HIRSCH AND AHRENS[67] free fatty acids were quite cleanly separated in a peak between the triglyceride and cholesterol peaks. Fatty acids in hydrolysates can readily be separated from more polar compounds[193–195].

The usual effects of chain length and unsaturation (see above)[196] allow at least partial resolution of mixtures of stearic, myristic, and lauric acids[1, 10, 11], and stearic and oleic acids[15]. More remarkable (*cf.* methyl esters, above) is the effect of geometrical isomerism: *trans*-oleic (elaidic) acid moves faster than *cis*-oleic, and *trans*-erucic (brassidic) faster than *cis*-erucic[9]. Hydroxy acids are eluted late[61, 62, 193, 197].

Quinones

It seems that Coenzymes Q (II) are eluted just after triglycerides and free fatty acids, and that at least some fractionation among them (different values of n) is possible[198].



(II)

Eluents that have been used are chloroform–hydrocarbon mixtures[198–200] and 2–5% ether in ligroin[201].

Among the lipids of *Mycobacterium tuberculosis* two naphthoquinone derivatives have been found, one (K_x) eluted in triglyceride fractions and the other (K_y) in a fraction containing monoglycerides[190, 202].

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The use of silicic acid chromatography in isolation of plant quinones (*e.g.* see ref.[203]) deserves mention.

Sterols

In chromatography of animal lipids cholesterol is eluted after triglycerides as the next major fraction[3,22,57,60,67,72,92,98,111,112,128,139,151,152,167,204], which is separate even when an unnecessarily polar eluent such as chloroform is used[98,111,112]. A preferable eluent is 10% ether in ligroin[22,60,67,92,139,140,151,152,167,204], several column volumes of which are needed because the cholesterol peak "tails"[67]. Coprostanol (saturated) can be eluted before cholesterol[180] and β-sitosterol[67] (monoenes); 7-dehydrocholesterol[180] (see Table I) and 24-methylenecholesterol[205] (dienes) are presumably eluted next, followed by ergosterol (triene), which BARRON AND HANAHAN[128] could not resolve from diglycerides.

NEHER'S review[101] gives useful information about more complex steroids.

Diglycerides

Since KAUFMANN[7,9] demonstrated the inverse order of adsorption of simple glycerides, tri- > di- > mono-, diglycerides have quite often been isolated chromatographically[14,21,67,128,186,188,189,204,206,207]. They can be eluted with 20–60% ether in ligroin or with benzene–chloroform mixtures[21,188]. Resolution of the diglyceride peak after the cholesterol peak is impaired by column overloading[67,128].

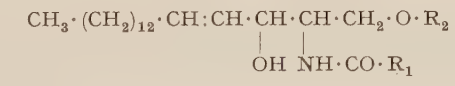
It is reassuring (contrast monoglycerides) that D- and L-1,2-diolein and their tetrabromides[206], and also D-1,2-dicaproin[207], are not isomerized on silicic acid columns. Fractionation of diglycerides seems feasible since distearin tends to be eluted before dipalmitin and dilaurin[67], and 1,3-dipalmitin moves slightly faster than 1,3-diolein on silicic acid-impregnated glass fibre paper (Table I).

Monoglycerides

Most monoglycerides can be eluted with ether or chloroform[21,67,128,138,186,188,190,204], but monoacetin requires a more polar eluent[67]. 2-Monoglycerides tend to be converted to 1-monoglycerides on silicic acid columns[21,67]: thus no separation of the isomers is possible.

*Glycolipids**

In 1956 WEISS[95] first reported silicic acid chromatography of cerebrosides which he eluted with a continuous, convex concentration gradient of methanol in chloroform. It seems clear that kerasin (III, $R_1 = (CH_2)_{22}\cdot CH_3$, R_2 = galactose residue) is eluted by chloroform alone[95,96,208,209] and phrenosin (III, $R_1 = CH(OH)\cdot(CH_2)_{21}\cdot CH_3$,



(III)

* Lipids containing bound sugars.

R_2 = galactose residue) by *ca.* 10% methanol in chloroform[95,96,197,208–210] or by acetone[211]. Phrenosin (sulphatide also[212]) precedes phosphatidylethanolamines and most other phospholipids[126,211–213]. But other, less well characterized cerebrosides[197,210–216] and gangliosides[95,216] are eluted with phospholipids. Cytolipin H (III, $R_1 = CH(OH)\cdot(CH_2)_{21}\cdot CH_3$, R_2 = glucosylgalactose residue?) is eluted before phosphatidylethanolamines[217,218]. A glucose-containing cerebroside from flies is eluted after phosphatidylethanolamines but before lecithins[111].

According to Cleland and Kennedy[209], "Dihydrosphingolipids are eluted only slightly more rapidly than their unsaturated analogues". Psychosine (galactosylsphingosine) is eluted by *ca.* 20% methanol in chloroform, and DNP-psychosine forms a peak between those of kerasin and phrenosin[209].

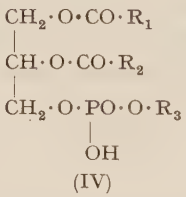
Cord factor from *Mycobacterium tuberculosis* (trehalose 6,6′-dimycolate) is eluted by 20% methanol in ether[61,63] (see also refs.[62,219]) and its methylated derivative by benzene–ether (1:1)[63]. Synthetic compounds related to cord factor have been purified by silicic acid chromatography[64,65], as have other complex glycolipids of mycobacteria[219–223] and plants[150].

Cytidine diphosphate-dipalmitin is eluted by *ca.* 20% methanol in chloroform[224].

*Lipoamino-acids**

Phosphatidylserines (IV, $R_3 = CH_2\cdot CH(NH_2)\cdot CO_2H$) are generally eluted in the phosphatidylethanolamine fraction[72,97,98,111–113,150,174,213,226–239].

Marinetti, Erbland and Stotz[85] took a fairly pure sample of phosphatidyl-



(IV)

serine isolated by non-chromatographic means, and chromatographed it on silicic acid. Eluting with chloroform–methanol (4:1 and 7:3) they obtained two main lipid peaks, the first of which consisted of free phosphatidylserine and the second of its sodium salt; these gave spots with identical R_F values when developed with an acidic solvent on silicic acid-impregnated filter paper. Apparent tailing of phosphatidylserines on columns[97,115,227,230,234–237,239] can be ascribed to this phenomenon. Under certain conditions it appears that silicic behaves as a cation exchanger, liberating phosphatidylserines from their salts[83]. Since phosphatidylserines can readily be separated from other phospholipids on silicic acid-impregnated filter paper (*e.g.* see ref.[240]) it seems that a good separation by column chromatography may sometime be achieved.

DNP-phosphatidylserines can readily be separated from DNP-phosphatidylethanolamines[241].

* For definition see ref.[225].

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The nature of tissue lipoamino-acids other than phosphatidylserines is still obscure[225], but it is very striking that they are found in minor quantities in most fractions eluted after cholesterol[98, 111–113, 150, 215, 220, 225, 228, 242–247]. Some are eluted by chloroform or ether without added methanol[98, 112, 222, 225, 247].

A synthetic phosphatidyltripeptide (IV, $R_1 = R_2 = (CH_2)_{16}\cdot CH_3$, $R_3 = CH_2\cdot CH(NH_3^+)\cdot CO\cdot NH\cdot CH_2\cdot CO\cdot NH\cdot CH_2\cdot CO_2^-$)[248*] is eluted specifically by 25% methanol in chloroform[115], and its derivative containing phenyl, benzyl and benzyloxycarbonyl protecting groups is eluted by ether[248].

Bile acids

Bile acids have been detected in eluates of blood[112] and liver[128] lipids. Hirsch and Ahrens[67] eluted lithocholic acid in the monoglyceride fraction, followed by desoxycholic acid. Wooton[249] has described chromatography of bile acid methyl esters.

Glycerophosphatidic acids

Phosphatidic acids (IV, $R_3 = H$) have a high R_F value on silicic acid-impregnated filter paper[240, 250–252], and are presumably eluted from columns before phosphatidylethanolamines[227, 253]. Similar behaviour is shown by the ill-characterized polyglycerophosphatidic acids[111, 234–238, 244, 254–257], including cardiolipin[182, 192, 258], although some may be eluted later[238]. Vilkas and Lederer[221] have eluted a mycobacterial phosphatidic acid as its magnesium salt.

Inositol-containing lipids

Phosphatidylinositols (IV, R_3 = 1-inosityl) can be eluted, apparently in salt form, after phosphatidylethanolamines[72, 84, 97, 114, 234, 235, 238, 239, 246, 255, 259, 260]. However, inositol phospholipids have been found eluted before or with phosphatidylethanolamines[150, 182, 228, 237, 261, 262] or as late as lecithins[98, 111, 112, 261]. This suggests the existence of new lipids, and it may indicate that free phosphatidylinositols are eluted earlier than the salts (*cf.* phosphatidylserines).

Phosphatidylethanolamines

Phosphatidylethanolamines (IV, $R_3 = CH_2\cdot CH_2\cdot NH_2$) are most commonly eluted with *ca.* 20% methanol in chloroform[24, 84, 97, 110, 182, 211, 226, 228, 234–239, 255, 263–267] (although methanol concentrations of 15% or below are sufficient[57, 72, 98, 111, 112, 150, 268]), and occasionally with 25% ethanol in hexane[229], 25% methanol in ether[92], or dichloromethane–methanol (13:7)[174]. Rhodes and Lea[226] and Wallach *et al.*[110] have clearly shown that the phosphatidylethanolamines first eluted are the most unsaturated. The reason for this behaviour, which is contradistinct from that of non-phospholipids, is not known; perhaps it reflects the degree of unsaturation in only one fatty acid residue (IV, R_1 or R_2).

Synthetic L-α-(dioleyl)-phosphatidylethanolamine has been eluted with chloroform–methanol (4:1), and its phthalimide with ether–methanol (4:1)[267].

* A sample was generously provided by Professor E. Baer.

Lysophosphatidylethanolamines

Lysophosphatidylethanolamines are eluted just before[111, 115, 182, 237, 265] and with[111, 115, 182, 226, 265] lecithins.

Lecithins

Lecithins (phosphatidylcholines; IV, $R_3 = CH_2 \cdot CH_2 \cdot N^+(CH_3)_3 \text{-}OH$) can be eluted slowly with as little as 20% methanol in chloroform[98, 115, 226, 269], but usually higher methanol concentrations are employed[24, 67, 72, 84, 97, 98, 111, 112, 150, 182, 194, 211, 230, 231, 234, 235, 238, 239, 264, 265, 268, 270–273] and occasionally mixtures of other liquids[174, 228, 229, 263, 274, 275]. Many workers[112, 174, 211, 230, 231, 234, 235, 238, 239, 262, 264, 268, 276, 277] have found it difficult to resolve the lecithin peak from the sphingomyelin peak that follows, but it is possible[57, 67, 72, 97, 182, 228, 263, 265, 273], and is facilitated by continuous concave gradient elution[98]. This method prevents the tailing characteristic of tissue lecithins. On silicic acid-impregnated glass fibre paper L-α-(dipalmitoleyl)-lecithin moves faster than L-α-(distearyl)-lecithin (see Table I; but note that a mixture could not be resolved), and the most unsaturated lecithins are eluted first from columns[97, 226] (*cf.* phosphatidylethanolamines).

Sphingomyelins

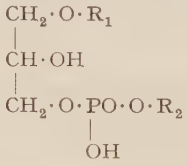
Sphingomyelins (III, $R_2 = O \cdot PO(O^-) \cdot O \cdot CH_2 \cdot CH_2 \cdot N^+(CH_3)_3$) can be separated in a peak between lecithins and lysolecithins[228, 230, 231, 278]. A concave concentration gradient of 30–50% methanol in chloroform elutes them[98].

Lysolecithins

Eluents used for lysolecithins range in polarity[24, 226, 230, 231, 235, 236, 239, 271] from 50% methanol in chloroform[265, 269] to methanol containing water[228, 263, 278]. Apparently silicic acid catalyzes acyl migration, 2-(acyl)-lysolecithins changing to the 1-[195, 265, 279], although TATTRIE AND MCARTHUR[269] may have separated 1- and 2-(palmityl)-lysolecithins, eluting the 2- first. On silicic acid-impregnated glass fibre paper (Table I) 2-(palmitoleyl)- moves faster than 2-(stearyl)-lysolecithin.

Ethers

"If an ether linkage replaces one of the carboxyl ester linkages in a triglyceride, the pattern of elution is unchanged"[67]. Thus, compounds such as chimyl dioleate are eluted with triglycerides[67, 138, 185]. Ligroin–ether (17:3) elutes the benzyl ether of D-1,2-dicaproin[207]. Chimyl (hexadecyl) and batyl alcohols (octadecyl D-1-glyceryl ether) are eluted with monoglycerides[67, 140, 185, 194]; 2-glyceryl ethers are more readily eluted than 1-glyceryl ethers[195, 266]. Glyceryl-1-phosphate-3-ethers (V, $R_2 = H$) can



(V)

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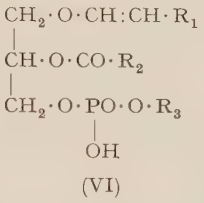
be eluted with 20% methanol in chloroform (chloroform alone is insufficiently polar) and the ether analogue of lysophosphatidylethanolamine (V, $R_2 = O \cdot CH_2 \cdot CH_2 \cdot NH_2$) with 55% methanol[197,210].

It has not yet been possible to separate ethanolamine plasmalogens (VI, $R_3 = CH_2 \cdot CH_2 \cdot NH_2$) from phosphatidylethanolamines[57,81,98,111,112,182,194,214,266,280] or choline plasmalogens (VI, $R_3 = CH_2 \cdot CH_2 \cdot N^+(CH_3)_3{}^-OH$) from lecithins[57,98,111,112,182,194,234,239,265,266,273,280]. It is claimed that the former separation can be achieved



(VI)

on silicic acid-impregnated filter paper at low temperature[236]. Ethanolamine lysoplasmalogens can be eluted with 50% ethanol in hexane[81].

Being vinyl ethers, plasmalogens are very reactive in presence of catalytic quantities of acids or heavy metal ions. Thus it is not unreasonable to suspect losses by hydrolysis during chromatography. GRAY AND MACFARLANE[182] showed indeed that some plasmalogen was hydrolyzed when a sample of whole ox-heart lipid was chromatographed in chloroform, followed by chloroform–methanol. After this experiment the column was exhaustively washed with chloroform and a second sample of the same lipid was chromatographed on it: plasmalogen was not then hydrolyzed. When material eluted by chloroform–methanol was introduced to a new column, in chloroform–methanol solution, hydrolysis was not detected. The interpretation made of these findings was that limited hydrolysis of plasmalogens occurs on silicic acid only in presence of the "fat" fraction. Another interpretation, not excluded by the findings reported, is that silicic acid catalyzes hydrolysis before, but not after, contact with methanol.

Miscellaneous lipids

α-Tocopherol[67,92,152,281] and its acetate[92] are eluted at about the same stage as cholesteryl esters (see Table 1) but after the synthetic antioxidant 2,6-di-*tert.*-butyl-*p*-cresol[282]. Numerous pigments of animal[98,110,174,232,283] and plant[150,284] tissues are eluted before phosphatidylethanolamines. Propylene glycol mono- and distearates can be eluted with chloroform[285], bis(distearyl-L-1-glyceryl) phenyl phosphate with ligroin–ether (4:1)[248], ceramides (III, $R_2 = H$) with 5% methanol in chloroform[112,197,210], sphingosine with chloroform–methanol (*ca.* 10:1)[209,286], DNP-sphingosine with $>$ 30% chloroform in benzene[209], and sphingosine hydrochloride with methanol[155].

Finally, some lipids of unproved structure will be mentioned. A primary aromatic palmitamide derivative in the unsaponifiable fraction of human liver has been isolated by elution with 40% ether in pentane[140]. Sirenin ($C_{21}H_{36}O_7$?), the sex-attractant of

Allomyces, is eluted by chloroform–methanol (99:1)[287,288]. An "unnaturally biosynthesized" diethanolamine lipid is eluted at the same stage as phosphatidylethanolamines[289]. N-Mono- and N,N-dimethylethanolamine lipids of *Neurospora* are eluted in the phospholipid fractions[290].

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For an Addendum to this article

see pp. 177–181

INORGANIC PAPER CHROMATOGRAPHY

A PROGRESS REPORT

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1. INTRODUCTION AND SCOPE

This review is intended as a discussion of the development of inorganic paper chromatography in the last three and a half years (1957 up to the middle of 1960). The previous literature has been reviewed by the author in the book by LEDERER AND LEDERER[1], and the bibliography by HAIS AND MACEK[2] also covers the work up to 1956. Since then this topic has not been reviewed in the English language.

The main emphasis of the review will be on new topics, such as modified filter papers, new separations and new applications. Of the 250 odd papers that have been published, a large number contain only slight alterations or particular applications of previous work. These will only be mentioned or, in some cases, omitted entirely. Since the important new R_F tables have already appeared in the section on "Chromatographic Data" of the *Journal of Chromatography*, R_F values will not be given in this review.

A number of Japanese, Russian and Polish publications were not available in the original and therefore their subject matter was taken from the *Bulletin Signalétique du Centre National de la Recherche Scientifique.*

2. ADSORPTION CHROMATOGRAPHY OF INORGANIC SUBSTANCES ON CELLULOSE

In this section we shall discuss a topic that has been rather neglected, *viz.* the separation of substances with aqueous solutions as developing solvents.

In some cases the exact process by which the substance is held on the paper is still open to discussion; however, we shall divide the various separations according to the probable interaction between the cellulose and the inorganic ions to be separated. In certain cases there seems to be no definite reaction with the cellulose, but rather an insolubilisation on the paper. These we have combined under the heading precipitation.

(*a*) *Precipitation*

Easily hydrolysable metal ions such as Sb(III) remain on the original spot when developed with water[3], while other ions can be precipitated by suitable reagents and thus one ion is held back while others separate through adsorption along the paper.

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The silver group (Ag^+, Pb^{2+} and Hg_2^{2+}) was separated in this fashion by developing first with water and then with ammonia[4], or by developing with alkali chlorides or HCl[5]. IMAI[6–8] worked out a scheme of analysis for most groups of metals, using such procedures. The copper group is first developed with ammonia (Pb^{2+} and Bi^{3+} at $R_F = 0$ and Cu^{2+}, Hg^{2+} and Cd^{2+} at the liquid front) and then the lower spot is separated with 0.4 N H_2SO_4 (Pb^{2+} $R_F = 0$, and Bi^{3+} at the liquid front) and the upper spot with 0.5 M K_2CO_3–0.3 M KI whereby Cd^{2+} stays at $R_F = 0$, Hg^{2+} moves with an $R_F = 0.6$ and Cu^{2+} is at the liquid front.

Similarly, As–Sb–Sn are separated with ammonium carbonate after oxidising As(III) to the pentavalent state (oxidation with HCl–$KClO_3$ and reduction with $NaNO_2$)[7] and Fe–Al–Cr are separated[8] with aqueous NaOH after oxidation of Cr (III) to CrO_4^{2-}.

PICKERING[9] investigated the movement of numerous metal salt solutions when developed with N KNO_3 with small amounts of HNO_3 added. The most important feature in such chromatograms is the formation of a definite front of acidity which moves behind the liquid front.

Movement on the paper of soluble ions away from insoluble ones has found applications in several radiochemical problems. PEREY[10] and ADLOFF[11] isolated francium from other radioelements by development with ammonium carbonate. Francium moves with the solvent while AcX and other radioelements of the Ac series are precipitated on the point of application.

Movement on the paper on development with aqueous alkali was taken as a criterion of the formation of alkali-soluble species (protactinates) of Pa(V). When Pa(V) is merely mixed with aqueous alkali it stays on the point of application; it is only after fusion or treatment with 10 N KOH that tracer ^{233}Pa will move with the liquid front[12,13].

CROUTHAMEL AND FUDGE[231] separated some fission products with 49% aqueous hydrofluoric acid.

There seems to be little difference between this kind of paper chromatography and the well-known capillary separations of microchemistry, except that development along paper strips is practised here instead of spotting on small squares of paper.

(*b*) *Cation exchange*

Radiotracer studies by BRODA, SCHÖNFELD and their collaborators[14,15], as well as equilibrium studies with very dilute solutions by PICKERING[16–18] show that filter paper behaves as a weak cation exchanger.This is no doubt due to the gluconic acid residues in the cellulose. The adsorption obeys the law of mass action unless hydrolysis interferes.

The cation exchange capacity of cellulose is, however, rather small. The most successful separations based on ion exchange have been achieved by developing tracer amounts with lactic acid (STRAIN *et al.*[19]).

(*c*) *The adsorption of anions*

RIPAN *et al.*[20–22] studied the formation of polyanions by chromatography on cellulose with aqueous solvents. For example the R_F value of molybdate in saturated KCl

varies from about 0 to 1 depending on the pH of the solvent (R_F = 0 at pH 0.2 and R_F = 1.0 at pH 8.5). Several spots are formed at intermediate pH values. Saturated KCl also proved a useful solvent to distinguish between dichromate (R_F = 0.66) and chromate (R_F = 0.92) and for distinguishing the products when phosphomolybdate is formed with different ratios of P:Mo, as well as for silicomolybdates with different ratios of Si:Mo. In all cases spots of low R_F value are the main products with high ratios (*e.g.* 1:12), while they have high R_F values with low ratios. Saturated KCl as well as water was also used as solvent in studying the reactions of molybdate, phosphomolybdate and silicomolybdate with varying amounts of alkali and $Ba(OH)_2$. In all cases the polymerised ion is adsorbed while the monomeric ion moves near the liquid front.

The question how these complexes are adsorbed was investigated by PLUCHET AND LEDERER[23]. Of about twenty inorganic ions examined in acetate buffers, only chromate, molybdate, tellurite (actually a cation) and vanadate have R_F values below 0.8. Neither the brand of paper nor its porosity nor the concentration of the anion chromatographed have an influence on the R_F value of the anion. An increase in the electrolyte concentration (sodium acetate) of the solvent causes the R_F values to decrease a little. The pH value in the range from 5.8 to 4 has no pronounced effect except on vanadate (which seems to change its polymeric state in this range). Anion exchange is thus unlikely and salting-out equilibria also. We should like to draw attention to the fact that all the adsorbed anions, in addition to forming polymers, are known to form complexes with vicinal OH pairs, as would certainly also occur in the cellulose network (for complexes of this kind see refs.[24–26]). Thus complexation with the paper is one possibility for the adsorption of anions.

(d) Adsorption in analogy to solvent extraction

KRAUS *et al.*[27] have recently shown that cation exchange resins, or even resins without active groups, may still adsorb chloro-anions of the type $AuCl_4^-$. This kind of adsorption on porous resins was already known for organic compounds[28,29]. Recently it was shown that cellulose also adsorbs chloro-complexes of the type $AuCl_4^-$ or $GaCl_4^-$ from hydrochloric acid solution. Salting-out agents lower the R_F values in the same manner as on resins (or as in ether extractions)[30]. This work was extended[31] and numerous ions were investigated, of which, however, only Ga(III), Au(III), Po(IV) and Sb(V) were found to be appreciably adsorbed from chloride or bromide solutions. Typical results are shown in Fig. 1.

Pentavalent antimony separates into two spots, one usually strongly adsorbed, the other at the liquid front. The latter seems to consist of one or more hydrolysed species, as its amount depends on the acid concentration of the starting solution (see also MATSUURA[100]).

The mechanism may be interpreted in various ways. The cellulose network can be considered as a polymerised trihydroxy-diether and the solvent action of such a surface made responsible, in which case we are dealing with a type of inversed phase chromatography. Or, which actually amounts to the same, we may consider the ether

oxygens as ionised oxonium ions and the chloro-anions as being retained by what can be called a form of ion exchange.

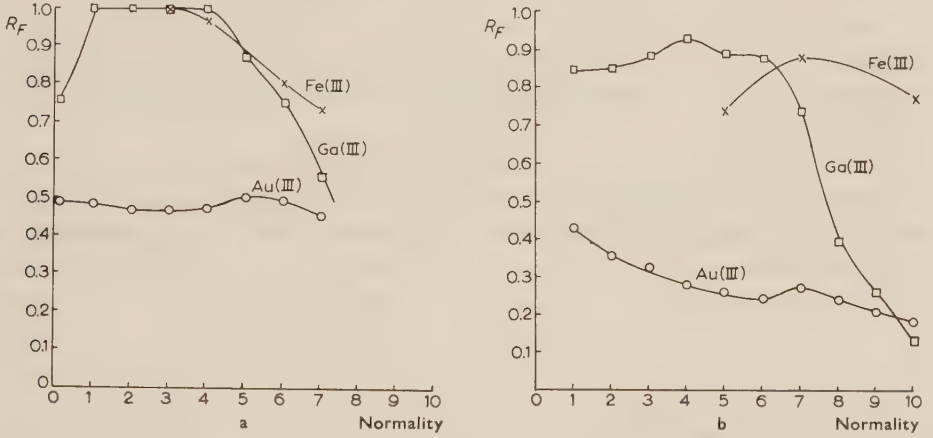

Fig. 1. The change of R_F value with the concentration of (a) HCl or (b) LiCl when the metal ions Au(III), Ga(III) and Fe(III) are developed with aqueous HCl or LiCl (containing 1% HCl to prevent hydrolysis).

(e) Adsorption from concentrated hydrochloric acid

FOUARGE AND DUYCKAERTS[32] have shown that separations of alkaline earths and some rare earths are possible on filter paper when 12 *N* HCl is used as the solvent. These authors point out that in 12 *N* HCl there are only 3.6 moles of water per mole of HCl and that the vapour pressure of concentrated HCl shows that the HCl is far from being completely hydrated. Thus here we are dealing with an inorganic solvent which has some similarity to organic solvents.

It seems doubtful whether development with concentrated hydrochloric acid will find analytical applications since the paper becomes rather fragile.

(f) Adsorption of metal complexes with organic ligands

It will be noted that in the above discussions only relatively few metal ions and anions were found to be adsorbed from aqueous solutions. The same observation was made with a large range of metal complexes, *e.g.* acetyl-acetonates, thiocyanates, dipyridyl complexes, etc., usually move with the liquid front. Exceptions are thiourea and rubeanic acid complexes which are strongly adsorbed from dilute hydrochloric acid solutions. A detailed study of some thiourea complexes[33] showed that the R_F values decrease with the amount of thiourea in the solvent and increase with the concentration of HCl in the solvent. Unstable thiourea complexes (*e.g.* Bi(III) and Pb(II)) have high R_F values, and stable complexes (*e.g.* Ru, Pd, Rh) have low R_F values. At the moment it is suspected that the adsorption mechanism has something to do with the sulphur in the ligand molecule.

The separation of thio-anions of As, Sb and Sn by MOHAN RAO[34] seems to be based on a similar adsorption mechanism. The thiourea complexes of Re and Tc were

studied by paper adsorption chromatography[35], and also the behaviour of the various Cu(I)–thiourea complexes described in the literature[36].

(*g*) *Peroxy-complexes*

AGRINIER[37] was able to isolate vanadium from soils by developing with an ammoniacal solution of hydrogen peroxide which separates the vanadium specifically.

(*h*) *Adsorption chromatography with fused salts on glass fibre paper*

A preliminary note by ALBERTI AND GRASSINI[38] describes some separations on glass fibre paper developed with fused salts. On using eutectics with low melting points the only complication is that the developing vessel must be placed in a thermostatically controlled oven.

3. SEPARATIONS OF INORGANIC SUBSTANCES ON MODIFIED FILTER PAPERS

(*a*) *Filter paper impregnated with ion exchange resins*

The preparation of filter papers loaded with cation or anion exchange resins was recently taken up by various firms (Rohm and Haas, U.S.A., Schleicher und Schüll, Germany, Balston, England). Although the papers available at present have not yet reached perfection (for example the Schleicher und Schüll papers contain too little of the resin) it is hoped that the laboratory methods for preparing resin papers (*e.g.* HALE[39], LEDERER[40] and LEWANDOWSKI AND WITKOWSKI[41]) will no longer be required in the near future.

The separation of trivalent from tetravalent metal ions was worked out on Dowex-50 papers, using 0.5 *N* to 3 *N* HCl[42]. For example Zr–Al separate with 2 *N* HCl, and UO_2^{2+}–Fe^{3+}–Th^{4+} with 0.5 *N* HCl.

Alkali metals were separated with 0.1 *N* HCl by ARNIKAR AND CHEMLA[43], and bismuth and uranium by PETERSON[44].

Dowex-50 papers also proved useful in determining the charge on cationic Rh(III) chloro-complexes[45,46]. For example, in 0.1, 0.2, 0.3 and 0.5 *N* HCl a complex of the type $Rh(H_2O)_5X^{2+}$ has the following R_F values: 0.09, 0.27, 0.46 and 0.75, while the hexaquo rhodium(III) ion stays at the point of application.

Separations of anions were recorded by LEWANDOWSKI AND IGNASIAK[47] who separated Cl^-–NO_3^-–SO_4^{2-}. SMILLIE[48] employs ion exchange resin papers in an ingenious micromethod for desalting solutions prior to the paper chromatography of organic substances.

(*b*) *Papers with ion exchange groups*

The literature on the preparation of these papers and most of the early work on inorganic separations has already been reviewed[1].

The commercial production of ion exchange papers by the manufacturers of Whatman papers should give a great impetus to work with such papers. Three cationic papers, a cellulose phosphate, a carboxymethyl-cellulose and a cellulose citrate paper as well as two anionic papers, an aminoethyl-cellulose and a diethylaminoethyl-

cellulose paper are available. The possibilities of such papers as a medium for two-dimensional analysis have been shown by KNIGHT[49], who used partition chromatography in one direction with organic solvents (butanol–HCl or acetone–HCl) and ion exchange chromatography with aqueous solvents (aqueous $MgCl_2$) in the second.

(c) Filter paper impregnated with a liquid anion exchanger

Solutions of tertiary amines in neutral organic solvents are now extensively employed in solvent extraction and have given rise to the expression "liquid anion exchangers".

TESTA[50] applied the use of such liquid ion exchangers to paper chromatography by impregnating the paper with the amine (tri-*n*-octylamine) and developing with aqueous hydrochloric acid. The numerous examples tested showed that such paper behaves for all purposes like a paper impregnated with a strong anionic resin. For example the mixture Zn–Mn–Ni separates with 10 *N* HCl (R_F values: Zn = 0.17, Mn(II) = 0.50 and Ni = 0.96). A separation of *zirconium* and *hafnium*, which seems to be better than any previously obtained on paper, was recorded. In 8 *N* HCl – 5% conc. HNO_3 hafnium has an R_F of 0.50 while zirconium has one of 0.17.

Both hydrochloric and sulphuric acid were studied for numerous separations of transition metals, uranium, thorium and zirconium.

(d) Filter paper impregnated with inorganic ion exchangers

Preliminary notes were published by PEIXOTO CABRAL[51] and ALBERTI AND GRASSINI[52]. It is important to precipitate the inorganic ion exchanger inside the paper. For example paper strips are first dipped into a solution of $ZrOCl_2$, dried and then dipped into a 60% solution of H_3PO_4 in 4 *N* HCl. Such papers behave like columns of zirconium phosphate. ALBERTI AND GRASSINI[52] list the R_F values for numerous ions in HCl of varying concentration.

Zirconium molybdate and zirconium phosphate papers prepared by CABRAL[51] served to show that separations of alkaline earths and of Cs from Ca–Sr–Ba proceed as on the corresponding columns.

In another paper ALBERTI AND GRASSINI[53] describe a separation of the alkali metals on ammonium phosphomolybdate paper.

(e) Asbestos paper

During work on the electrophoretic separation of isotopes it was observed that the ion exchange properties of asbestos sheets were sufficient for chromatographic separations. The separation of alkali metals was carried out and compared with separations on Dowex-50 paper (ARNIKAR AND CHEMLA[43]). See also ARNIKAR[54]. ARNIKAR AND MEHTA[55] describe a separation of radioactive Y, Ba and Sr.

(f) Acetylated paper

The water-repellent properties of acetylated papers make development with aqueous solvents impossible. With organic solvents there is an inversion of the movement for some of the chloro-complexes as shown in Table 1.

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TABLE I

R_F VALUES OF SOME METAL IONS ON WHATMAN NO. 1 PAPER AND BINZER ACETYLATED (43% ACETYLATED) PAPER
(B. KLINKEN AND M. LEDERER, to be published)
Solvent: butanol–conc. HCl (2:1)

Metal ion	R_F	
	Whatman No. 1	*Binzer 43% acetylated*
Au(III)	1.0	0.20–0.28
Bi(III)	0.53	0.58
Cd(II)	0.89	0.65
Cu(II)	0.62	0.55
Fe(III)	1.0	0.23
Hg(II)	0.86	0.63
Tl(III)	1.0	0.35
Pd(II)	0.60	0.59
Sb(III)	0.76	0.41
Se(IV)	0.64	0.66
Ni(II)	0.14	0.18
Ga(III)	1.0	0.17
Sb(V)	1.0 + comet	0.15 + forward comet
Al(III)	0.07	0.16
Co(II)	0.63	0.63
Mn(II)	0.15	0.28

(g) *Filter paper impregnated with reagents*

In a series of papers NAGAI[56–60] describes separations of mixtures of transition elements by chromatography on paper impregnated with 8-hydroxyquinoline. YOKOTA AND SHIMADA[61] separate Fe–Cu–Mn–Ni on paper impregnated with kojic acid. A separation of the copper group metals on paper treated with dithizone was also described by NAGAI[62]. There is little difference between this kind of chromatography and the classical capillary separations on impregnated papers, usually classified under spot tests.

(h) *Chromatography on impregnated viscose fibres*

TANAKA[63] describes the separation of Sr, Ca, Zn and Y on viscose fibres dyed with either Brilliant Blue Japanol 6BKX or Rapid Scarlet Benzo 4BS. Isopropanol–aqueous 80% NH_4CNS (200:25) is recommended as solvent.

4. PARTITION CHROMATOGRAPHY OF INORGANIC SUBSTANCES ON PAPER

A. Separations of metal ions

(a) *General*

The details of washing the paper (with HCl and water) were dealt with by LU MING-LIEN *et al.*[64]. ORESHKO AND GENSEROVSKAJA[65] studied the effect of large amounts of

sugars in the sample on the separation of metals in acetone–HCl–water. Cu is slowed down when sugars are present while Fe(III) is not affected.

COCH-FRUGONI[66] pointed out that the sequence of Cd–Bi can be altered in butanol–HCl–water depending on the degree of saturation of the paper and the atmosphere with the solvent. When the system is equilibrated Bi precedes Cd; under non-equilibrium conditions this is reversed. GHE AND PLACUCCI[67] found a relation between the R_F value and the dielectric constant of the solvent (in the case of aliphatic alcohols) for Mn, Co and Cu.

(b) Schemes of qualitative analysis

The scheme of POLLARD *et al.*[68] has been extended by ELBEIH AND ABOU-ELNAGA[69] to include Li, Be, Ce, Ti, V, Mo, Th and U.

Two new schemes based on group separation and paper chromatographic separation of the groups have been proposed.

PFEIL *et al.*[70] separate the H_2S group on Binzer 202 paper with butanol–3 *N* HCl by ascending development (R_F values: Ag^+ = 0.0, Cu^{2+} = 0.22, Pb^{2+} = 0.32, Sb(V) = 0.39, Au(III) up to 0.40, Ge(IV) = 0.49, Te(IV) = 0.56, Mo(V) = 0.62 (and a second spot at 0.87), Bi(III) = 0.65, Se(IV) = 0.73, As(V) = 0.83, Cd^{2+} = 0.85, Sn(IV) = 0.92 and Hg(II) = 0.94).

The platinum metals are also separated in butanol–3 *N* HCl (R_F values: Os = 0.93, Pt = 0.88, Pd = 0.76, Ir = 0.32, Ru = 0.30 and Rh = 0.30). There are some possible sources of error in these separations, for example Sb(V), Ir, Ru and Rh can give more than one spot as mentioned also on pp. 136, 144, 145, 152.

For the ions of the ammonium sulphide group the solvent acetic acid–pyridine–HCl (80:6:20) is employed (R_F values: Fe(III) = 0.92, Zn(II) = 0.82, Co(II) = 0.68, UO_2^{2+} = 0.68, Mn(II) = 0.57, WO_4^{2-} = 0.43, Ni(II) = 0.39, VO_3^- = 0.35, Al(III) = 0.30, Cr(III) = 0.26 and Ti(IV) = 0.20).

The ammonium carbonate group and the alkali metals are separated with methanol–acetic acid (98:2) (R_F values: Be = 0.88, Mg = 0.74, Ca = 0.66, Sr = 0.52, Ba = 0.39, and Ra = 0.26, Li = 0.66, Na = 0.48 and K = 0.32).

BLASIUS AND GÖTTLING[71] worked out a more detailed scheme, which, however, necessitates separation into ten subgroups before the chromatographic separations. Radial development is used throughout.

Group 1. Precipitation with HCl yields the group Ag, Pb, Hg(I) and Tl(I); the chlorides are then dissolved in a solution of NH_4CNS–NH_4Ac and developed with 10% aqueous ammonium acetate. R_F values: Pb = 0.95, Ag = 0.80, Tl = 0.75 and Hg = 0.65.

Group 2. The solution left from group 1 is concentrated and when 2 *N* with HCl, it is reduced with hydrazine chloride which precipitates Au, Se, Te and Pt (partially). These are dissolved in HNO_3–H_2O_2 and developed with ethanol–2 *N* acetic acid. R_F values: Au = 1.0, Se = 0.60, Pt = 0.50 (0.90) and Te = 0.0.

Group 3. The filtrate from above is evaporated with conc. HCl and the sulphides precipitated with 2% thioacetamide. The arsenic group is then dissolved from the

mixed sulphides with 0.5 ml 0.5 *N* KOH (10 min on the water bath). The copper group sulphides are washed, dissolved in HCl–H_2O_2 and developed with butanol–acetone–water–acetylacetone -HNO_3 (49:33:10:5:3). R_F values: Hg = 1.0, Bi = 0.65, Cu = 0.35, Cd = 0.25 and Pb = 0.15.

Group 4. The arsenic group is developed with ethyl acetate–methanol–10% aqueous oxalic acid (68:12:20). R_F values: Sb = 1.0, Sn = 0.80, As = 0.50 and Mo = 0.40 (Pt = 0.20).

Group 5. The filtrate from the sulphide precipitation is evaporated with conc. HNO_3. Then the HNO_3 is removed by evaporation with HCl and ethanol is added for the reduction of MnO_4^- and CrO_4^{2-}. Phosphate and tungstate must be removed by passing over an ion exchange column. For vanadate (only if present) an excess of Fe(III) is added. After the removal of interfering anions, the solution is neutralised with ammonia and ammonium carbonate and the hydroxides precipitated by warming with urotropine. The precipitate is washed and dissolved in HCl, excess 30% NaOH and 3% H_2O_2 are added until the titanium group is precipitated. This group is then chromatographed with ethanol–conc. HCl (83:17). R_F values: Zr = 0–0.05, Ce = 0.05–0.15, Ti = 0.55 and Fe = 1.0.

Group 6. The solution containing CrO_4^{2-}, vanadate and uranium is reduced with SO_2 in alkali, boiled, and the precipitate washed with alkaline SO_2 water. As solvent for this group acetone–butanol–HCl–acetylacetone (33:43:19:5) is used. R_F values: Cr = 0.30, V = 0.50 and U = 0.80.

Group 7. The filtrate from the hydroxide precipitation contains Al and Be, which are precipitated after neutralising with HCl and developed with methyl ethyl ketone–HCl (85:15). R_F values: Al = 0.30 and Be = 0.55.

Group 8. The filtrate from the urotropine precipitation is made ammoniacal and freshly prepared $(NH_4)_2S$ solution added drop by drop and heated to 40°. The precipitate is washed with an NH_3–NH_4Cl solution and dissolved in HCl–H_2O_2. The group is developed with methyl ethyl ketone–HCl (91:9). R_F values: Zn = 0.85, Co = 0.60, Mn = 0.30 and Ni = 0.05.

Group 9. The filtrate from the ammonium sulphide precipitation is treated in the usual way to destroy the excess ammonium salts and urotropine. The alkaline earths are precipitated with ammonium carbonate with addition of ethanol. The solvent used is methanol–HCl–water (80:10:10). R_F values: Mg = 0.80, Ca = 0.55, Sr = 0.35 and Ba = 0.20.

Group 10. The filtrate is evaporated to remove the ammonium salts and the residue is dissolved in HCl and developed with the same solvent as for the alkaline earths. R_F values: Li = 0.80, Na = 0.40 and K = 0.20.

This scheme was given in more detail as it seems to be sound. However, it cannot be claimed to simplify the usual micro-qualitative schemes.

(*c*) *Solvents*

An important advance was made in inorganic paper chromatography by the systematic investigation of large numbers of metal ions in numerous solvent systems.

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The first, by GUEDES DE CARVALHO[72], deals with the R_F values of sixty-odd metal ions in butanol–aqueous HCl (1:1) mixtures, in which the concentration of the HCl varied from 2 *N* to 12 *N*. MAJUMDAR AND MUKHERJEE[232] list R_F values in butanol–water–HCl (70:20:10).

The system acetone–aqueous HCl (90:10) was investigated for about twenty metal ions, with the concentration of HCl ranging from 3 *N* to 12 *N* [73].

MAJUMDAR AND CHAKRABARTTY[74] give the R_F values of 27 metal ions in acetone–water–HCl (40:10:5).

HARTKAMP AND SPECKER[75–77] investigated the R_F values in the system tetrahydrofuran–water–HCl, varying all three constituents over wide ranges. They explain the behaviour of metal ions by considering the hydration, solvation and chlorocomplexation equilibria and derive essentially the same correlation as was found for the system acetone–HCl–water[73]. See also BOUMANS[78].

A more recent paper by GUEDES DE CARVALHO[79] also gives the R_F values of about sixty metals in the butanol–water–HCl system, with variation of all three components.

VERNOIS[80] recorded the R_F values of metal ions likely to form fluoride complexes, such as Fe, Ti, Nb, Ta, Pa, Zr, Hf and U, in the system butanol–water–HCl–HF, in which, on the one hand, the concentration of HF was varied from 0 to 25% for butanol–6 *N* HCl (1:1) and, on the other, the concentration of HCl was varied from 3 *N* to 12 *N* for mixtures with butanol (1:1) containing 1% of HF. GRAND-CLEMENT *et al.*[81] list R_F values for acetone–HCl–HF–water (90:5:1:4), butanol–HCl–HF–water (100:50:2:48) and butanol–H_2SO_4–water–HF (100:9.5:85.5:5) for fifty metal ions.

With the main purpose of providing data for solvent extraction KERTES *et al.* recorded R_F values of numerous metal ions for the following systems: methyl isobutyl ketone–HNO_3[82], methyl isobutyl ketone–HNO_3–$LiNO_3$–water[83], di-isopropyl ether–nitric acid–water[84], methyl isobutyl ketone, di-isopropyl ether and iso-amyl alcohol with NH_4CNS and water, with or without addition of HNO_3 to the system[85], iso-amyl alcohol–nitric acid–water[86].

Tributyl phosphate containing nitric or hydrochloric acid was investigated by WEIDMANN[87], WEIDMANN AND DRABNER[88] and KERTES AND BECK[89].

For the separation of complexes of 1-nitroso-2-hydroxy-3-naphthoic acid with several metals see DATTA AND GHOSE[90].

(*d*) *General reagents for cations*

Several new general methods for the detection of cations have been proposed. None seems to be a notable improvement on the previously known reagents. SINGH AND PURSHOTTAM[91] suggest the use of diphenyl-violuric acid instead of the already well-known violuric acid. The colours can only be obtained when the paper is weakly acid, *i.e.* when solvents containing acetic acid are used. Chloranilic acid, proposed by BARRETO AND BARRETO[92], seems to have the same shortcomings as the previously mentioned reagent. A table of the sensitivities of the reaction is given in the original paper.

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LEWANDOWSKI[93] suggests two reactions for the detection of cations. The first consists in spraying with sodium carbonate followed by sodium sulphate and an indicator. Wherever carbonates of the metals are formed there must be a decrease of the pH value. The second reaction involves spraying with ferrocyanide and precipitation of the insoluble metal ferrocyanides followed by a $CuSO_4$ or $FeCl_3$ spray which shows up the unreacted ferrocyanide as an intensely coloured background. These reactions as well as many others, are possible on paper but work only in certain concentration ranges; the spot to be detected should have the cation in excess of the sodium carbonate or the ferrocyanide, otherwise no distinct contrast will be observed.

(e) The separation of groups of metals

In addition to the schemes of analysis described on pp. 141, 142 a number of papers deal with separations of groups of metal ions.

(i) The silver group: Pb^{2+}, Ag^+, Tl^+ and Hg_2^{2+}. YAMADA[94] proposes a new solvent system, acetone or methyl ethyl ketone containing 40-50% of perchloric acid. When the insoluble chlorides are dissolved in excess KI, ethyl acetate–ammonia–acetone (10:2:5) is used. CELAP AND RADIVOJEVIC[240] recommend methanol–11 *N* HNO_3 (60:40) (R_F values: $Ag^+ = 0.29$, $Tl^+ = 0.39$, $Pb^{2+} = 0.51$, $Hg_2^{2+} = 0.75$).

MAJUMDAR AND CHAKRABARTTY[95] list the R_F values of Ag^+, Tl^+ and Pb^{2+} in 18 solvents, some showing promise of separation.

(ii) The copper group: Cu, Pb, Cd, Bi and Hg. MAJUMDAR AND CHAKRABARTTY[95] also indicate R_F values for the copper group in 18 solvents. A new solvent containing caprolactam was suggested by SIKORSKA-TOMICKA *et al.*[96]. ALMASSY AND DEZSÖ[97] separate copper from numerous elements with mixtures of water–ethanol–ethyl ether–HCl. For the separation of cadmium see also ZIEGLER AND WINKLER[98].

(iii) The arsenic group: As, Sb and Sn. BARNABAS AND BARNABAS[99] record separations with acetic acid–acetone–water (8:1.5:2.5) (R_F values: As = 0.59, Sb = 0.76 and Sn = 0.92).

MATSUURA[100] described the behaviour of Sb(V) on paper chromatograms developed with butanol–HCl. Sb(V) exists usually as an equilibrium mixture of oxychloro-complexes and $SbCl_6^-$. While the oxychloro-complexes have low R_F values, $SbCl_6^-$ moves usually near the liquid front. Unless the oxidation of Sb(III) is carried out in conc. HCl and the solvent is very rich in conc. HCl several spots are invariably obtained.

For R_F values of Sb and Sn see also MAJUMDAR AND CHAKRABARTTY[96].

(iv) The iron and aluminium group elements. The separations of these elements are dealt with in conjunction with the rarer elements such as vanadium, gallium, etc.

(v) The alkaline earths. DE VRIES *et al.*[101] employ radial development with HCl–methanol–tetrahydrofuran (70:20:10); the reagent oxine may be added to the solvent and does not affect the separation. MODREANU *et al.*[102] use ethanol–water–propionic acid–ammonia (20:2:1:1) as solvent. See also MODREANU[103]. For a separation of beryllium see ALON AND ADER[104].

(vi) The alkali metals. SCHILLER AND TÖLGYESSY[105] chromatographed Na^+ and K^+

as iodides to which ^{131}I had been added and thus obtained a quantitative estimation of the two alkalis by radioactivity measurements. MODREANU[106] developed the alkalis with ethanol–water–propionic acid–ammonia (100:10:5:5), detected the spots with violuric acid and determined the amounts by the spot area method. MARTIN[107] found that the solvent methanol containing 1% to 5% ammonia has the advantage that the spots of the alkalis can be detected with indicators. See also BOUZKOVA *et al.*[108], BARRETO AND BARRETO[109], NAKANO AND SHIMADA[138] and, for a separation of K, Rb and Cs as the picrates, MODREANU *et al.*[110].

(f) The separation of rarer metals

(i) The platinum metals. Two papers by MAJUMDAR AND CHAKRABARTTY[111,112] require some comment. In both papers the solutions of the metals studied are referred to as "solutions of the chlorides of Pd(II), Pt(IV), Ir(IV), Rh(III), Ru(III), Os(IV), Au(III)". In the first paper solvents containing butanol–HCl or butanol–acetic acid with admixtures of KI, and acetone solvents with or without KI were used for development. The reviewer fails to understand how in most solvents no reduction comet of Ir(IV) to Ir(III) was observed. Also the results with Rh(III) are unreliable unless the exact method of preparation of the solution is specified, because in most cases multispots due to the presence of several aquo-chloro-rhodium(III) complexes will be formed[113,114].

In the second paper[112] it must again be regretted that the authors do not mention the preparation of the solutions as this makes the work impossible to repeat. In the various solvents containing thiourea, the formation of complexes is tacitly assumed; however, it is well known that ruthenium does not form the blue complex with quite a variety of ionic species present in solutions and when it is formed, heating for several minutes is necessary. The reviewer also found that heating was required to convert Rh(III), Pt and Pd to their thiourea complexes[33].

(ii) Other rare elements (Ga, Ge, In, Tl, Zr, etc.). *Gallium* was separated from Fe, Al and Ti by SLAMA AND LADENBAUER[115] with butanol–acetic acid–ethyl acetate–water (50:10:5:35). MAGEE AND SCOTT[116] list the R_F values of *Al, Ga, In* and *Tl(III)* in eight different mixtures of phenol–methanol–conc. HCl. *Vanadium, molybdenum* and *tungsten* were separated by SHIH-FU *et al.*[117] in solvents containing H_2O_2.

Separations and quantitative estimations of *germanium* were described by NAGY AND POLYIK[118] and PINHEIRO DICK[119]. *Zirconium* and *thorium* were separated by LEBEZ AND PIRS[120] with acetone–5% aqueous oxalic acid (100:26). For a separation of uranium from numerous metal ions with ether–ethanol–HNO_3 (70:23:7) see ALMASSY AND VIGVARI[121]. MORACHEVSKI *et al.*[122] separate U–V–Fe with a mixture of acetic acid–nitric acid.

(iii) Niobium and tantalum. BLASIUS AND CZEKAY[123] separated Nb–Ta with organic solvents containing oxalic acid (*e.g.* acetone–2 *N* HCl–10% aqueous oxalic acid (80:10:10)). Similar work was also reported by CABRAL[124]. LEDERER AND VERNOIS[125] and VERNOIS[80] obtained good separations of Ta–Nb in their study of the paper chromatography of protactinium with butanol–HCl–HF. See also ref.[81].

(iv) The rare earths, scandium and thorium. A separation of *uranium* and *cerium*

was described by CVJETICANIN AND BELEGISANIN[126], a separation of thorium from many elements by ALMASSY *et al.*[127].

DANON AND LEVI[128,129] found that a butanol solvent containing both HNO_3 and $LiNO_3$ (butanol–aqueous 7 *M* $LiNO_3$ + 2 *M* HNO_3 (80:20)) gave separations such as Ac–La or La–Y–Sc, also several pairs of rare earths such as La–Ce, Ce–Nd, Pr–Eu, but usually a bad resolution of adjacent rare earths. LAUER AND POLUEKTOV[130] employed acetone–ether mixtures containing thiocyanate for separations of rare earths with subsequent determination with complexones. MAZZA[131] reports several separations both by paper chromatography and paper electrophoresis.

(*v*) *The separation of different valencies of the same element.* STEVENS[132] published separations of the different valency states of Mo, V, Co, Fe (see also ref.[133]), Mn and Cu. From another paper by STEVENS[134] we shall mention some of the details of the separation of Mn(II), Mn(III) and MnO_4^-. In a mixture of acetic acid–acetic anhydride (10:30) on acid washed Whatman No. 1 paper the R_F values are: Mn(II) – about 0.16, Mn(III) = 0.94 and MnO_4^- = 0.48. Cu(I) and Cu(II) were separated in a mixture of ethanol–acetic acid–ether (30:5:50) in an atmosphere of CO_2.

For the separation of Fe(II) and Fe(III) see also POLLARD *et al.*[135].

B. Separations of anions

(*a*) *General*

A scheme of analysis of anions, along the lines of the differential spraying method of POLLARD AND MCOMIE, has recently been proposed by ELBEIH AND ABOU-ELNAGA[136]. Only one solvent, ethanol–pyridine–water (60:20:16), is used. After development the chromatogram is cut into two segments for spraying with numerous reagents. TAMURA AND MIYAZAKI[137] separate Cl^-, Br^-, I^-, CNS^-, ClO_3^-, BrO_3^-, NO_3^- with acetone–water (9:1), F^-, NO_2^-, $S_2O_3^{2-}$, SO_4^{2-}, CrO_4^{2-}, IO_3^-, AsO_3^{3-}, BO_2^-, ferro- and ferricyanide with butanol–methanol–water (1:3:1) and oxalate, arsenate and phosphate with butanol–2 *N* HNO_3.

NAKANO AND SHIMADA[138] utilise paper chromatography and paper electrophoresis in a scheme of analysis which also divides the anions first into groups. Butanol–ammonia–collidine (3:1:1) is used to separate Cl^-, Br^-, I^-, CNS^-, CN^-, ferro- and ferricyanide. For a separation of CrO_4^{2-}, MoO_4^{2-}. WO_4^{2-} and VO_3^- see MURATA[139].

(*b*) *Borates*

NUNES DA COSTA AND GUEDES DE CARVALHO[140] attempted the separation of polyborates but obtained only one spot in a large range of solvents. The equilibria between the various borates seem to be instantaneously reversible.

(*c*) *Nitrogen acids*

STEVENS[141] separated hyponitrite, $N_2O_2^{2-}$ from nitrite, nitrate and hydroxylamine with ethanol–water–KOH (70:30:2) as solvent. Hyponitrite has a low R_F value (0.1) while the values of all the other nitrogen compounds are in the region 0.5–0.6.

References p. 153/157.

(d) Phosphorus acids

The review by HETTLER[142] in Volume 1 of this series discusses the problems of the separation of polyphosphates in detail and lists 66 references on the topic of phosphorus acids.

Since the review by HETTLER, the following papers concerning condensed phosphates have appeared: BERG[143], BERNHART AND CHESS[144,145], HEINERTH[146], MITCHELL[147] and SMITH[148]. SCHMULBACH, CHESS AND BERNHART[149] discuss specifically the detection of traces of pyrophosphate in orthophosphate. For separations of fluorophosphates and fluoroarsenates, see KOLDITZ *et al.*[242,243].

(e) Sulphur acids

A simple method for the detection of sulphate on paper chromatograms, developed with butanol–HCl, consists in heating the paper in an oven at 105°. Charring is produced with sulphate, nitrate and phosphate (COCH-FRUGONI[150]). LEHMANN AND KEMPE[151] detect all sulphur acids by oxidation with N_2O_3 and then spraying with Pb^{2+}. The $PbSO_4$ is detected on the paper with Na_2S after removing the excess Pb^{2+} ions. They also list R_F values of imidosulphate, amidosulphate, sulphamide, etc. in dioxane–water–1% ammonia (75:24:1).

SERVIGNE AND DUVAL[152] and GARNIER AND DUVAL[153] studied the movement of polythionates, sulphate, hydrogen sulphate, pyrosulphate and persulphate. In butanol–water only persulphate ($R_F = 0.36$) and pyrosulphate ($R_F = 0.57$) move from the point of origin. In isopropanol–water (75:25) both sulphate and hydrogen sulphate yield two spots since in a neutral solution they are in equilibrium. STEGER AND SEENER[154] separate the *thiophosphates* obtained from the hydrolysis of P_4S_7, using isopropanol–water–trichloracetic acid (80:20:5) as solvent. The work on polythionates by BIGHI *et al.*[155–158] is a continuation of the previous studies by these authors. For an application to the study of the reaction of H_2S with SO_2, see BARBIERI AND BRUNO[159,237]. JAKUBOVAC AND PROCHAZKA[160] chromatographed elementary sulphur.

(f) Selenium and tellurium acids

SCHNEER AND ÖRDÖGH[161] use paper chromatography for studying the oxidation of tellurite to tellurate.

(g) Halogen acids

HALL[162] worked out a paper chromatographic method for the detection and determination of microgram amounts of fluoride. A new reagent for fluoride was proposed by LEWANDOWSKI AND SZCZEPANIAK[163]. The paper is sprayed with a suspension of $Al(OH)_3$ which complexes the fluoride and then with an indicator which shows the pH change due to the complexation.

ZIPKIN *et al.*[164] separate fluoride from phosphate with methanol – 2 *N* NH_4OH (70:30) (R_F values: $F^- = 0.65$ and $PO_4^{3-} = 0.34$).

SERVIGNE[165] and LAUB[166] describe the separation of the halogen oxyacids.

References p. 153/157.

(*h*) *Technetium and rhenium*

A number of R_F values of pertechnetate and perrhenate in acid and alkaline solvents were recorded by LEVI AND LEDERER[167], who employed a long-life ^{99}Tc isotope to improve on previous work carried out with a short-lived ^{99*}Tc tracer. GUEDES DE CARVALHO[168] made an extensive study of the lower valencies of Tc and Re. Potentiometric titrations with $SnCl_2$ at various acidities were carried out and samples withdrawn at certain intervals and chromatographed. Thus it was possible to indicate the change of R_F value with the concentration of HCl in butanol, even with valencies which dismutate considerably during development.

C. Quantitative methods

Only the papers dealing specifically with quantitative estimations after paper chromatographic separation will be mentioned in this section. Numerous other semiquantitative and quantitative methods are mentioned in conjunction with specific separations, etc.

(*a*) *Retention analysis*

Although this method does not yield more than semiquantitative results it was proposed by YAMADA[169] for the determination of Cu on chromatograms.

(*b*) *Colorimetry*

POLLARD *et al.*[170] carried out extensive work on the separation of mixtures of heavy metals (Tl, Ag, Zn, Cd, Co, Ni, Cu, Fe, Bi, U, Hg) especially with lead and worked out a number of colorimetric procedures with extremely accurate results. Synthetic mixtures of 1 part of the trace metal in 500 parts of lead, for example, yielded an accuracy of $\pm$ 4% for Zn, Fe, Cu or Bi.

LACOURT AND HEYNDRYCKX[171] use colorimetry for the determination of Co and Zn and note that the lower limit of detection is 0.03 μg for Co and 0.02 μg for Zn.

(*c*) *Titration*

Direct titration without elution from the paper has been shown to yield highly accurate results with micro amounts. This method which was developed by LACOURT is described in several detailed discussions[172–176]. Two others workers, ELBEIH AND ABOU-ELNAGA, worked out microtitration methods using EDTA as the titrant: uranium[177], thorium[178], Pb, Bi, Fe, Mn, Mg with EDTA and Be with oxine[179], Cu and Ni[180], Ba with EDTA, uranium with ferrocyanide[181].

D. Analytical applications

In most of the preceding sections no indications were given as to the applications of the chromatographic separations. In many cases they are evident, in others the chemical problems are indicated. In Table 2 we have listed those papers which deal with specific analytical applications.

References p. 153/157.

TABLE 2

ANALYTICAL APPLICATIONS OF INORGANIC PAPER CHROMATOGRAPHY

Material analysed or type of analysis	*Metal ions detected*	*Reference*
(1) *Geochemical prospecting*		
Minerals	Ag, Ni, Co, Cu, Nb, Ta, Ti	AGRINIER[182]
Minerals	Li, B, Be	AGRINIER[183]
Minerals	Mo	AGRINIER[184]
Minerals	Be	AGRINIER[185]
Minerals	U	JEDWAB[186]
Minerals	U	SUKANOUE[187]
Natural waters	U	WARD AND MARRANZINO[188]
(2) *Trace metal analysis*		
Edible fats	heavy metals	TÄUFEL AND ROMMINGER[189]
Latex of hevea	Ca, Mg, Al, Cu	BERNAL AND BOUCHER[190]
Flotation concentrates	Ge	GHE[191]
Refrigeration sludge	metal ions	STEVENS AND BOPP[192]
Volatilisation products of oxide cathodes	Ba, Sr	DUSING AND KUNZE[193]
Calcium salts	Ba and Sr	ISHIBASHI AND TSUTSUI[194]
Tungsten and cobalt alloys	Fe	LIANG YU-CHEN[195]
Nickel	Co	KRAUSZ AND MOLNÁR[196]
Alloys	Mn, As, Cr	HARASAWA AND AIHARA[197]
Copper	Au	HARASAWA AND HAYANO[198]
Cobalt	Fe, Cu, Ni, Zn, Pb	DE ANDRADE DIAS AND PINTO COELHO[199,200]
Ferric oxides and ferrites	metal ions	BOVALINI AND PIAZZI[201]
	Co, Ni, Cu and Zn	FRIERSON *et al.*[202]
Biological materials	Cu	KRZECZKOWSKA[203]
(3) *Forensic and pharmaceutical analysis*		
Forensic samples	Tl	KLIFFMÜLLER[204]
Toxicological analysis	Cd, Zn, Pb, Bi, Tl, Cu, Hg	NITSCHKÉ[205]
Forensic analysis	metal ions	PFEIL AND GOLDBACH[206]
Pharmaceutical products	Cu and Co in presence of much Fe	STERESCU AND KEIM[207]
Pharmaceutical products	$HgCl_2$	SADOK[208]

E. Paper chromatography in the study of metal complexes

(a) Cobalt(III) complexes

STEFANOVIC AND JANJIC[209] examined a large number of pairs of *cis*- and *trans*-Co(III) complexes and conclude that as a rule the *cis*-complex has a higher R_F value than the corresponding *trans*-complex.

POLLARD *et al.*[210] studied the nitro-ammino and the ethylenediamine Co(III) complexes. A large number of ether–methanol–water–HCl mixtures were tried as solvents and the ratio 50:30:20:2 found useful for the separation of ammine complexes.

(b) Nitroso-ruthenium complexes

The separation of these complexes requires great speed as they decompose during development. In one paper WAIN *et al.*[211] suggest methyl isopropyl ketone–*N* HNO_3

as solvent, in another [212] dibutyl cellosolve. Methyl isopropyl ketone gives the following R_F values:

Trinitrato-Ru-NO	1.0
Dinitrato-Ru-NO	0.6–0.8
Mononitrato-Ru-NO	0.2–0.35
Polynuclear species	up to 0.1

See also BROWN[213] and FLETCHER *et al.*[238].

(*c*) *Ferrocenes*

DE BELDER *et al.*[214] separated ferrocene, acetylferrocene, ferrocenealdehyde, hydroxymethylferrocene and ferrocenecarboxylic acid using paper impregnated with dimethyl sulphoxide and as solvent light petroleum saturated with dimethyl sulphoxide.

(*d*) *Ferric phosphate complexes*

BARBIERI *et al.*[215] separate the following complexes and free ions in butanol–HCl–water (87:8:5): $NaH_5[Fe(PO_4)_3]$, $R_F = 0.03$; $Na_3[Fe(PO_4)_6]$, $R_F = 0.20$; $Na_6[Fe_2(P_2O_7)_3]$, $R_F = 0.16$; Fe^{3+}, $R_F = 0.48$; PO_3^-, $R_F = 0.80$; PO_4^{3-}, $R_F = 0.80$; $P_2O_7^{4-}$, $R_F = 0.74$.

(*e*) *Organo-metallic compounds*

A number of organo-mercury compounds such as phenylmercury acetate and ethylmercury chloride were separated by KANAZAWA *et al.*[241]. WILLIAMS AND PRICE[239] published a list of R_F values of organo-tin compounds in pyridine, butanol–pyridine–water, butanol–NH_3–water and butanol–ethanol–water.

(*f*) *Complex equilibria in paper chromatography*

Most separations in organic solvents containing mineral acids are based on differential complex formation. It is possible to correlate the change in R_F value with the change in the amount of the complexant. For example when in a solvent, such as acetone, which does not solvate cations, the relative amounts of water and HCl are varied, it can be shown that for a given ion

$$-\mathrm{p}K = R_M + 2x\ \mathrm{pCl}^- + K$$

where $R_M = \log (1/R_F - 1)$, pK = the pK of the complexing reaction in the solvent, $\mathrm{pCl} = -\log (\mathrm{Cl}^-)$ and K is a constant[73].

Typical results for some metals in acetone containing 10% aqueous HCl are shown in Fig. 2.

An attempt to interpret the R_F values in butanol–acid solvents was made by KERTES[216]; however, here one difficulty is that butanol can also solvate cations although not to the same degree as water. If one assumes that an increase in the R_F value indicates complex formation, then comparison of R_F values in solvents containing different acids (but otherwise identical) will indicate the relative complexing ability of the acids. KERTES[217] used this approach to determine the complexing strength of mono-, di- and trichloroacetic acids. GUEDES DE CARVALHO[79] carried out extensive

work with the system butanol–water–HCl. The variation of R_M values with the increase of the amount of HCl or the amount of water in the butanol indicates whether either of these constituents influences the complex or hydrated state of the metal ion. See also p. 143.

SINGH AND DEY[218–224] published a series of papers on the development of mixtures of varying ratio of complexant and metal ions with a neutral solvent such as aqueous

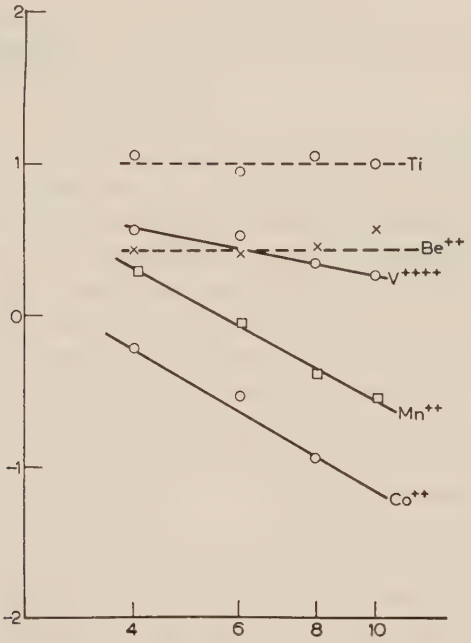

Fig. 2. The graphical representation of the variation of the R_M values of Ti, Be, V(IV), Mn(II) and Co(II) with the pCl^- of a mixture of 90 % acetone–10 % aqueous HCl.

alcohol. The movement of the metal ions depends of course on the ratio and at given ratios low R_F values or precipitation at the origin is recorded. Separation of binary and ternary mixtures is also possible with definite ratios so that one metal is complexed and the other not. The theoretical and practical applications of this work have been left unexploited so far.

F. The paper chromatography of radioelements

(*a*) *Natural radioelements*

LEVI AND DANON[225] separated Po from RaD, RaE, TeO_3^{2-} and SeO_3^{2-} in a solvent containing nitrate as the complexing agent instead of the usual chloride. In a mixture of butanol–propanol (1:1) saturated with an aqueous solution containing 7 *M* $LiNO_3$ and 2 *M* HNO_3 they record the following R_F values: RaD = 0.27, TeO_3^{2-} = 0.47, RaE = 0.62, SeO_3^{2-} = 0.72 and Po = 0.97.

CROUTHAMEL AND GATROUSIS[226] also report separations of Po from the above

elements with solvents such as ethanol–conc. HCl (6:1) or ethanol–isopropyl ether–HCl (2:1:0.5) or even as fluorides in a mixture of methyl ethyl ketone–49% HF (100:60). However none of these seem to be improvements of the usual separations in butanol–HCl (see ref. [1], page 528).

CONTE *et al.*[227] prepare pure RaE by developing first with aqueous ammonia to rid the solution of an excess of nickel introduced during its deposition on nickel foil, then with butanol–1 *N* HCl to remove traces of other radioelements. Several natural radioelements have already been mentioned in other sections of this review. For francium see p. 135 and refs.[10,11], for polonium see p. 136 and ref.[31], for radium see p. 141 and refs. [32,70].

(*b*) *Artificial radioelements*

A second paper by GÖTTE AND PÄTZE[228] deals with the separation of the fission products which are precipitated with H_2S (with Cu^{2+} carrier). Ruthenium yields up to six spots in most solvents and must be removed by distillation. The remaining longer lived isotopes are divided into two groups, those which coprecipitate with $Y(OH)_3$ or $Fe(OH)_3$ on addition of ammonia (Te, Sb and Sn) and those which remain in solution (Ag, Mo, Pd and Cd). Rhodium may be found in both groups.

The Te, Sb and Sn group has to be reduced with SO_2 and then yields a single spot for Sb and Sn, but Te is partially reduced to the element (solvent: ethanol–methanol–2 *N* HCl (3:3:4)). For the Ag, Mo, Pd, Cd and Rh group ethanol–methanol–2 *N* HCl (4:4:2) or ethanol–butanol–5 *N* HCl (3:3:4) gave good separations; however, rhodium yielded a double spot.

FOUARGE[229] and FOUARGE AND FUGER[230] investigated several solvents especially for the separation of the fission product alkalies and alkaline earths (Cs and Sr). Methanol containing small amounts of HCl and various mixtures of phenol–HCl were investigated in detail. See also FOUARGE AND DUYCKAERTS[32].

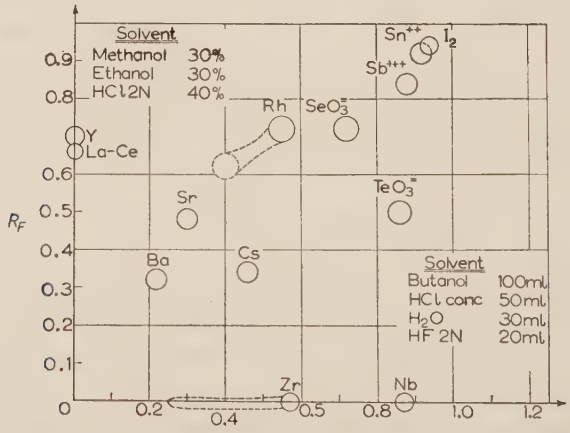

Fig. 3. A two-dimensional chart showing the distribution of the major fission products when chromatographed first with methanol–ethanol–2 *N* HCl (30:30:40) and then with butanol–HCl conc.–water–HF conc. (100:50:48:2).

CROUTHAMEL AND FUDGE[231] reported separations of fission product elements with aqueous 49% HF, methyl ethyl ketone–HF (100:20 or 100:60). It is of interest that the R_F values of Np, Pu and Am are indicated, being 0 in the first two solvents and in the last 0.2 for Np and Pu and 0 for Am.

Two-dimensional separations of the fission product elements were also described by GRAND-CLEMENT *et al.*[81], a distribution of spots as shown in Fig. 3 being obtained.

(c) Plutonium

A paper chromatographic study of plutonium by FINK AND FINK dating from 1949 has only been declassified recently[233]. A range of mixtures of methyl ethyl ketone with HNO_3 or HCl, also aqueous solvents and butanol solvents were investigated and tables of R_F values for numerous elements given. The R_F values for Pu(IV) for most of these solvents were also indicated, for example for butanol–1 *N* HNO_3 it is 0.0.

A preliminary account of the separation of the various valency states of plutonium is given by ENGLISH AND FOREMAN[234]. Pu(IV) and Pu(VI) could be separated in butanol–3 *N* HCl, Pu(III) and Pu(IV) with mixtures of ethyl acetate–conc. HCl. An oxidising solvent containing ethyl acetate, nitric acid and sodium nitrite, can be used to separate Pu(IV) from the higher actinides (which are trivalent).

G. Miscellaneous

CARTLIDGE AND TIPPER[235] separate *hydrogen peroxide* from organic peroxides. In butanol–ether–water (10:10:1) hydrogen peroxide has an R_F of 0.5, while hydroxyperoxides and hydroperoxides move near the liquid front. For the separation of most organic peroxides a glycol impregnated paper is used with petroleum ether–butanol (90:10) as the mobile phase. Here H_2O_2 has an $R_F = 0.05$.

POLLARD AND BANISTER[236] separate hydrazine, hydroxylamine, ammonium chloride and some organic derivatives of hydrazine and hydroxylamine with water–HCl–ether–methanol (15:4:50:50) as solvent.

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A COMPREHENSIVE BIBLIOGRAPHY OF RECENT SEPARATIONS OF INORGANIC IONS BY ELECTROMIGRATION IN PAPER*

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A large number of separations of inorganic ions by the technique of electromigration in paper have been reported over the past ten years. These are widely scattered throughout the literature, and no complete collection of them has appeared, although BLASIUS has given a good compilation of many of them[1]. It was felt that a thorough coverage of the inorganic separations studied by this method would be useful, particularly to those considering a practical application of the techniques for the first time. The following compilation is believed to be complete up to March 1960.

The various separations have been arranged according to an arbitrary classification. No system of grouping of the ions studied can be completely satisfactory. The ions considered in various separations have been chosen so widely that a good deal of overlap from one group to another is unavoidable. Often, the prime factor governing selection of the ions investigated seems to have been ease of detection.

Most separations have been accomplished by a discontinuous process utilizing migration along a strip of paper. A few have used electromigration in two dimensions in a rectangular sheet, and others have combined electromigration with paper chromatography in a two-dimensional procedure (electrochromatography). The latter appears in both continuous and discontinuous forms. Where the technique used differs from the common linear, discontinuous strip technique, this has been noted. No differentiation is made between the various forms of the latter procedure, *i.e.* hanging or horizontal strips, and open and closed strip operation. A separation by one such technique can be accomplished also by any other, although effects of evaporation and flow of the background electrolyte solution may modify the behaviour in some cases. Many partial separations which have been reported might readily be made complete if longer migration paths were used. A large amount of the published work gives migration distances of only a few centimetres. While a few voltage gradient and separation times are given in order to illustrate the results obtained, the same separation may be carried out in different times using any voltage gradient up to a

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maximum determined by the amount of heat which can be dissipated. These factors therefore are not important.

The work which can be considered historical, *i.e.* the small amount done prior to 1950, will not be covered here. It is dealt with by McDONALD[2], for instance. Some of the modern work in packed columns and gels is mentioned, but coverage of this technique may not be complete. The work of MANECKE on the electromigration of cations and anions in columns of cation and anion ion-exchange resins may be noted here[3]. The specialized "focussing" technique of SCHUMACHER, in which a complexing gradient is used, will not be dealt with in the following tables, although it has indeed given some striking results when applied to a number of metallic cations[4–8].

A few lists of relative zone mobilities of some ions have been published. These may be useful in indicating whether a proposed separation is feasible. Such values are given for anions in 0.1 *N* NaOH by GRASSINI AND LEDERER[9], in $(NH_4)_2CO_3$ by GROSS[10] and by LEDERER[11] (0.1 *M*, and 2% solutions respectively) and in 2% Na_2CO_3 by BELLING AND UNDERDOWN[12]. CETINI has given relative zone mobility values for cations in solutions of Na_2CO_3–lactic acid, HCl–potassium biphthalate, and HCl–sodium citrate[13], while MAKI has reported a few in electrolyte solutions made up with organic solvents[14].

Although actual separations are not included, the work of DE ANGELIS *et al.* on the variations of the mobilities of Co, Mn, Fe, Zn, Cu, Pb, Al, and Mg as a function of pH in 0.05 *M* citric acid, 0.01 *M* SCN^-, and KNO_3 solutions[15], and the studies by YASUNAGA AND SHIMOMURA on the effect of pH on very many anions and cations in $NaNO_3$, KCNS, and tartaric acid electrolytes[16] are of interest.

Most of the separations are presented below in tabular form. The ions studied and the background electrolyte solutions used are given, along with a few comments which may be useful. In some reports, such a large number of background solutions were employed that it is not practical to list them here; in some cases those yielding the best results are indicated.

I. THE ALKALI AND ALKALINE EARTH IONS

TABLE I

THE ALKALI AND ALKALINE EARTH IONS

Migrants	*Background electrolyte*	*Remarks*	*Ref.*
Mg, Li, Na, K	$(NH_4)_2CO_3$	Separated all in 35 min at 30 V/cm. K, Rb, Cs could not be separated. Relative mobilities are Mg(0.34), Li(0.53), Na(0.72), K(Rb,Cs) (1.00)	17
Mg, Li, Na, K, Rb, Cs	0.1 *M* $(NH_4)_2CO_3$	Separations at 100 V/cm. Complete in 37 min. Rb and Cs still very close together. Migration sequence (in order of increasing mobility): Mg, Li, Na, K, Cs, Rb	18

(continued on p. 160)

TABLE 1 (*continued*)

Migrants	*Background electrolyte*	*Remarks*	*Ref.*
Mg, Li, Na, K, Rb	NH_4OH	All but K and Rb separated	19
Na, K	NH_4Cl–HCl	Linear and radial techniques. A more fundamental study of the conditions for separations	20
Li, Na, K, Rb, Cs, Mg, Ca, Sr, Ba, Ra	HCl, lactic, tartaric and citric acids and their ammonium salts, EDTA at several pH values, and others	K, Rb, Cs could not be separated. Ba, Cs separated in di- and tri-ammonium citrate, but Sr overlapped both. In NH_3–EDTA solutions of pH 6, alkaline earths are anionic and easily separated from alkalis	21
Mg, Ca, Sr, Ba	0.05 *M* citric acid, pH 5.5	All separated in 45 min at 100 V/cm. Also some separations from group 3 and 4 ions	18
Mg, Ca, Sr, Ba	0.007 *M* citric acid in 5 *N* NH_4OH, and others	Electromigration, and discontinuous electrochromatography. Ions of groups 3, 4 and 5 were also studied	22
Mg, Na, K, and anions	Ethanol–acetic acid–ammonium acetate	Discontinuous electrochromatography. Most of separation due to chromatographic effects. Electromigration separated only cations and anions	23
Mg, Ca, Sr, Ba	0.1 *N* NH_4Cl, 0.1 *N* NH_4NO_3, and others	Sr and Ba could not be separated. Ternary mixtures separated in given electrolytes. Binary mixtures separated in other electrolytes	24
Mg, Ca, Sr, Ba	$Na_4P_2O_7$–HCl of pH 3.2	Sr, Ba have similar mobilities, but ternary mixtures not involving this pair were separated	25
Ca–Ba; Ba–La; Sr–Y; Sr–Ba	0.05 *M* lactic acid	Sr–Ba only partially separated	26
Sr, Ba	Methanol–acetone–HCl		14
Ca–Sr; Ca–Ba	HPO_3; pH 1.5		27
Ca–Sr	(HPO_3); pH 1.75	Also separated Ba from Zn, Pb, Fe, and Co, Al, Fe	27
Sr–Ba		Discontinuous electrochromatography	28
Ra from Pb, Bi, and Po	0.1 *M* lactic acid	Separation of Ra from its decay products	29
Sr–Y, Rb, Cs, Ca and others	0.1 *M* lactic acid	A few separations	29

Separation of the alkali metals by paper electromigration has been carried out by a few workers, but the difficulty in detecting these ions on paper has offered a considerable obstacle. Flame photometry has been used, but this is tedious. The alternative has been to use a background electrolyte such as ammonium carbonate which is easily volatilized, having an alkali metal compound which can be detected by an

acid-base indicator. Radioactive tracers, which offer the best means of detection, have so far seen little use.

The most difficult ions to separate of the alkali metals are K^+, Rb^+, and Cs^+. These have very similar mobilities. The separation of Li^+ and Na^+ from the rest and from each other is not difficult.

The alkaline earth ions are in many ways similar to the alkalis; the mobilities of Ca, Sr, Ba, and Ra are very similar. Each alkaline earth ion migrates more slowly than the corresponding alkali metal ion. Moreover, the former can form anionic complexes, thus giving a much wider scope for separatory procedures.

MACH has used this technique in determining the radiochemical purity of Na, K, Rb, and Sr of this group[30].

ARNIKAR[31] has studied the electromigration of alkali metals in asbestos paper at 350° with fused $NaNO_3$ as background electrolyte. Mobilities (10^{-4} $cm^2.sec^{-1}.V^{-1}$) are: Li (3.75), Na (4.16), K (3.74), Rb (3.58), Sc (3.38). Cs and K were not separated in 45 cm due to extensive low concentration regions behind and ahead of the main zones.

2. Sc, Y, THE RARE EARTHS, AND THE ACTINIDE ELEMENTS

TABLE 2

Sc, Y, THE RARE EARTHS, AND THE ACTINIDE ELEMENTS

Migrants	*Background electrolyte*	*Remarks*	*Ref.*
Eu–Pm–Ce	0.035 *M* tartaric acid–0.015 *M* diammonium tartrate	Eu, Pm anionic; Ce cationic. Separation accomplished	32
(Pr, Pm, Ce)–(Y, Nd)–Sc	0.1 *M* lactic acid	Separations accomplished. Ions in brackets had same mobilities	32
Sc, Sm, Nd, Y, Pr, Ce, La, Ac	Citric acid	Too short a migration distance for good separations to be achieved	33
Sc, Sm, Nd, Y, Pr, La, Ac	1% citric acid	As above	34
Ce, Y, U, Sr, Cs, Nb, Zr	0.1 *M* lactic acid	Continuous electrochromatography. Some ordinary electromigration separations also reported	29
Y, La, Ce, Pr, Nd	0.05 *M* NaCl, 0.125 to 0.05 *M* citric acid pH 2.6 or 3.05	At lower pH, all cationic; at higher pH, all anionic. Some ions separated in both instances	35
Sc, Y, La, Ce, Sm, Nd, Eu, Pr, Tb, Er	Acetic acid–oxine mixtures	A few separations accomplished, but electrolyte not as good as citric acid	36
Y, Ce, Pr, Nd, Th	Acetone–lactic acid	All separated in 90 min at 46 V/cm	37
Y, La, Ce, Pm ,Eu	Citric acid	Continuous electrochromatography in cell packed with quartz powder. A study of conditions of separation, with very promising results	38

(continued on p. 162)

TABLE 2 (*continued*)

Migrants	*Background electrolyte*	*Remarks*	*Ref.*
La–Ba; Y–Sr; Y–La	0.05 *M* lactic acid	Ba much faster than La. Sr much faster than Y. Y and La could be separated in a fairly long time	26
Th(IV), Pa(V), U (VI), Fe(III), Pb(IV), Bi(III), Zr (IV), Ta(V), Nb(V), Po(III), Ti (IV)	HF, and HF–HCl mixtures of various concentrations	Separated Fe, Th, U, Zr, Bi, and Pa; Fe, Pa, and Zr; and Pa–Zr, as well as others. Chiefly for purification of Pa	39
U, Pa, Ta, Nb, Zr, Ti, Fe	5% HF–5% HCl, and 1% HF	U, Pa, Zr separated in former, Pa, Fe, and Zr in latter electrolyte. Chiefly for purification of Pa	40
UO_2, Co, Fe(III)		Migrated as 'complexone' complexes in various background electrolytes	41
UO_2 and ions of groups 3, 4, 5, and 7	0.05 *M* citric acid, pH 3.1, and other values	Separated in 30 min at 140 V/cm	18
Th(IV), UO_2(II) and others	Many	Deals with the members of the third analytical group. See Section 3 and 7	42
UO_2, Th, Pu	0.1 *M* lactic acid	Separated as radioactive tracers. Pu formed long, diffuse zone, overlapping both others	29
UO_2 from Pt, Pd, Bi, and Fe	Many	UO_2 separated from the rest. Migration sequences given. Chiefly binary mixtures.	43

The rare earth elements and their homologues have such similar mobilities and chemical properties that separations by electromigration are quite difficult.

BACHELET *et al.* have given the direction of migration of UO_2(II) in many electrolytes, but no separations or quantitative information are given[44].

MAKI has separated the rare earths in monazite[45] by the method outlined in ref.[35]

3. Cr, Mn, Fe, Co, Ni, Zn AND Al

TABLE 3

Cr, Mn, Fe, Co, Ni, Zn AND Al

Migrants	*Background electrolyte*	*Remarks*	*Ref.*
Fe(III), Co(II), Ni(II)	Alcoholic KCNS–*N*/2 HCl	Separation readily accomplished	46
Co(II), Ni(II)	1 *N* HCl; 7% KCNS in 66% ethanol	Ni anionic, Co remains cationic. Separation as above	47

(continued on p. 163)

TABLE 3 (*continued*)

Migrants	*Background electrolyte*	*Remarks*	*Ref.*
Fe(III), Co(II), Ni(II), Zn(II), Al(III), Ba(II)	HPO_3, pH 1.5; $(HPO_3)_3$, pH 1.6 and 1.75	Separations from each other and from some copper and arsenic group ions. Quaternary mixtures considered	27
Fe(III), Zn(II), Co(II), Ni(II), Al(III), and the copper group	Many	Chiefly a separation of Fe(III) from the others. Only pairs of ions were separated	48
Zn(II), Mn(II), Co(II), Ni(II), and the copper group	Many	Zn and Mn separated from each other and the rest. Migration sequences given	49
Zn, Co, Ni, and the copper and arsenic groups	Many	Chiefly deals with the separation of members of the arsenic group from the others	50
Co, Ni, Ag, and the copper group	Many	Chiefly deals with the separation of Ag and Hg from many other ions, including Co and Ni	51
Fe(II), Fe(III), Co, Ni, Zn, Al	0.05 *M* citric acid at various pH values	High voltage separations from each other and other ions of groups 2, 4, 5, and 7	18
Co, Ni, and the copper group	Many	Separations from quaternary mixtures with copper group ions	52
Fe(III), Co(II), Al(III), and others	Many	Separations of these ions from some of groups 2 and 7 (the 3rd analytical group). Only a few separations of quaternary mixtures obtained	42
Ni, Zn, Co, Mn	KCN at pH 6.0. Many others tried	Co, Zn always formed overlapping zones in KCN. Complete separation of any 3 possible	53
Ni(II), Co(II), Mn(II), Zn(II), Al(III), Cr(III), Sn(II), Sb(V)	0.1 *M* ammonium tartrate in 4 *N* NH_4OH. Other proportions used also	Co–Ni–Mn–Zn, and Sb(V)–Sn(II)–Cr(III)–Al(III) were the groups separated	54
Fe, Al, and CrO_4^{2-} from As, Sb, Sn	$Na_4P_2O_7$– HCl, pH 2.4	Studied quaternary mixtures only	25
Mn, Co, Ni, Zn	Ethanol–acetone–HCl	Separation of Ni, Co, and Mn from Zn	14
Fe(II)–Fe(III)–Co(II)–Cd(II)–Pb(II)–Cu(II)	2 *N* acetic acid–0.6 *N* formic acid	Sequence (in order of decreasing speed): Fe(II), Co, Cd, Pb, Cu, Fe(III). Fe(II) and Co zones very close together	55
Fe, Ni, Co, Al, and some copper group ions		Discontinuous electrochromatography	28
Mn, Co, Ni, Zn, Fe, Al, CrO_4, Cu, Ag	Many	One- and two-dimensional techniques. Not all separated in one experiment	56

(*continued on p. 164*)

TABLE 3 (*continued*)

Migrants	*Background electrolyte*	*Remarks*	*Ref.*
Fe(III)–Co; Fe(III)–Mn; Fe(III)–Mg; Al–Tl–As(III)	0.05 *M* malonic acid–0.01 *M* acetic acid	Continuous separations using a tapered paper sheet	57
Fe(II), Ni, Co, Cu	1% NaK tartrate	Continuous electrochromatography of the α,α'-dipyridyl complexes. They are all anionic, sequence of mobilities Fe>Ni>Co>Cu. 1,10-Phenanthroline complexes also studied	58
Fe(II), Fe(III)	1% NaK tartrate	Continuous electrochromatography. Fe(II) as α, α'-dipyridyl complex, Fe(III) as acetylacetone complex	58
Fe(II)–Fe(III)	0.1 *N* H_2SO_4 and 0.1 *N* H_2SO_4–glycerol (1:1)	Separation in 90 min at 10 V/cm. Fe(II) faster	59
Fe(II)–Ni(II); Fe(II)–Cr(III); Fe(II)–Fe(III)	1% citric acid, and 0.5% NaK tartrate	Continuous electrochromatography. Fe(II) present as dipyridyl complex, $FePy_2$(II). Also separation of Fe(II)–Fe(III)–$FePy_2$	60
Fe(III), Co, UO_2		"Complexone" complexes in several electrolytes	41
Ni–CrO_4^{2-}; Ni–Cu	1 *M* acetic acid. 0.1 *M* lactic acid	Used for quantitative analysis after elution	61
Fe(III)–Ni; Ni–Ag; Ni–Cu; Co–Cu–Cd–Ag; Al–Co–Fe(III)	Several	Two-dimensional discontinuous technique; chromatography used in one dimension	62
Ni–Cu; Ni–CrO_4^{2-}; Fe(III)–Al; Fe(III)–Ni–Cu–Ag	Several	Continuous electrochromatography	62
Fe(III) from elements of groups 2 and 7	HF, and HCl–HF	See groups 2 and 7	39 40
Fe(III), Cr, Al, Mn, and ions of groups 1, 4 and 5	0.007 *M* citric acid in 5 *N* NH_4OH, and others	Electromigration, and discontinuous electrochromatography	22

Since these ions offer considerable scope for the use of complexing agents of various sorts, they have been investigated extensively.

In addition, separations of some precious metals from some of these ions are given by MUKERJEE[63–65] (see group 6).

CETINI[66] has separated some ions of this group and of groups 4 and 5 in a starch gel, using NaOH–potassium biphthalate and NaOH–lactic acid electrolytes. Best results were given by the former at pH 3.5. KAKIHANA *et al.*[67] separated Co and Cu by electromigration in a column packed with Amberlite 1RC–50 ion exchange resin.

References p. 174/176.

4. THE COPPER GROUP (Cu, Cd, Hg(II), Pb, Bi), AND Ag, Hg(I), Tl, Po

TABLE 4

THE COPPER GROUP (Cu, Cd, Hg(II), Pb, Bi) AND Ag, Hg(I), Tl, Po

Migrants	*Background electrolyte*	*Remarks*	*Ref.*
Cu, Cd, Bi; Hg(II), As, Cu	1 *N* HCl	Also separated Cu from Au, Pd, and Pt	68, 69
Cu, Cd, Pd, Bi, Hg(II), Tl(I), and elements of groups 2, 3, 5 and 7	0.05 *M* citric acid at various pH values	Ions separated from each other and from ions of other groups. A high voltage separation	18
Hg(II)–Pb–Ag	3 *N* NH_4OH; 30% formic acid; 0.05 *M* citric, lactic, acetic and tartaric acids	Could not obtain complete separations under the conditions used. Reported two zones formed by Ag in many of these electrolytes	70
Cu, Cd, Hg(II), Pb, Bi	0.1, 0.5, and 1.0 *N* HCl	Separation complete in 15 min at 12 V/cm. Cu cationic at all concentrations; Bi, Hg(II) anionic	46
Cu, Cd, Pb, Bi, Co, Ni	Many	Separations of quaternary mixtures	52
Cu, Cd, Hg(II), Pb, Bi	0.1 *N* KCl; several others	KCl gave best separation, but other electrolytes also gave separations	71
Hg(II), Pb, Ag, Tl(I)	1 *N* NaCl, KCl, KCN, and several others	Separations complete in 3 h. Other electrolytes failed to give complete separations	72
Cu, Cd, Hg(II), Pb, Bi	NaCl–HCl–glycine, pH 1.8	Bi motionless, Hg(II) anionic, remainder cationic	73
Cu, Cd, Pb, Bi, Ni, Fe(III)	HPO_3, pH 1.5; $(HPO_3)_3$ pH 1.6	Separations of some ions from group 3 as well as the copper group ions	27
Ag, Hg(II), Cu, Cd, Pb, Bi, Co, Ni	Many	Chiefly a separation of Ag and Hg(II) from the others	51
Cu, Cd, Pb, Bi, Ag, Fe, Be, Al, Zn, Co	Many	Separations of pairs only	48
Cu, Cd, Hg(II)	0.1 *N* KCl, HCl of various concentrations	Chiefly a study of migrational behaviour. Separations not given	74, 75
Cu, Cd, Hg(II), Pb, Bi	1, 2, and 4 *N* HCl	Excellent separations, but the high conductivity means that much heat is produced	76
Cu, Cd, Hg(II), Pb, Bi	KCl, KBr and HBr at various concentrations	Best separation in 0.5 *N* KBr, or 0.5 *N* HBr. As above, high conductance causes much heating, but separations in less than 1 h are possible. Much information given about the behaviour of halide complexes	77
Cu, Cd, Hg(II), Pb, Bi		One- and two-dimensional separations	56

(continued on p. 166)

TABLE 4 (*continued*)

Migrants	*Background electrolyte*	*Remarks*	*Ref.*
Cu–Ag; Cd–Ag–Bi–Al; Tl(I)–Al; Tl(I)–Al–As(III)	0.01 *M* EDTA in 1 *M* NH_4OH. 0.05 *M* ammonium malonate. 0.05 *M* citric acid, and others	Continuous separations	57
Cu, Cd, Pb, Co, Fe(III), Fe(II)	2 *N* acetic acid–0.6 *N* formic acid	See Section 3	55
Bi, Pb, and groups 2 and 7	HF and HF–HCl mixtures	See Section 2	39
Cu–Ni	0.1 *M* lactic acid	Used for quantitative analysis after elution	61
Cu–Cd	Aqueous acetic acid–pyridine	Cd faster than Cu	78
Ag–Pb–Hg(II); Cu–Cd–Pb–Bi–Hg(II)		Discontinuous electrochromatography	28
Ag–Ni; Cu–Ni; Hg(I)–Pb–Ag; Hg(II)–Ag; (Hg–Bi)–(Cu–Pb–Cd); Ni–Ag–Cd–(Cu–Co–Fe)	Many	Discontinuous electrochromatography. Ions in brackets formed overlapping zones	62
Cu–Ag–Ni–Fe	A basic tartrate–oxalate mixture. Others also used	Continuous electrochromatography. The electrolyte given separated all	62
Cu, Cd, Bi, Ag, Pb, Hg(II)	0.007 *M* citric acid in 5 *N* NH_4OH, and others	Electromigration, and discontinuous electrochromatography. Ions of groups 1, 3, and 5 considered also	22
Cu from Pd and Pt	1 *N* HCl	Cu is cationic, others anionic	79
Pb–Bi–Po–Ra	0.1 *M* lactic acid	Separation of Ra decay products including RaD, E, and F. Bi is adsorbed near the starting point, Po moves slowly as an anion. Po, Bi zones overlap somewhat	26, 29

The copper group ions have been studied quite extensively, since they form complexes with many reagents, and are all readily detected on paper. The other ions frequently have been considered with the copper group.

Separations of some precious metals from some of these ions have been given by MUKERJEE[63–65] (see group 6). Separation of Zn and Mn from these ions is given in ref.[49], and As, Sb, and Sn from the copper group in ref.[50]. Separation of Cu from Fe(II), Ni, and Co was given in ref.[58]. CETINI[66] has studied some ions of this group and of groups 3 and 5 in a starch gel, using NaOH–potassium biphthalate and NaOH–lactic acid electrolytes. LEDERER AND COOK[80] separated Cu, Bi, and Hg in a 2 % agar gel

with 0.5 *N* NaCl as electrolyte. The separation of Cu and Co in an ion exchange resin was accomplished by KAKIHANA *et al.*[67].

5. THE ARSENIC GROUP (As, Sb, Sn)

TABLE 5

THE ARSENIC GROUP (As, Sb, Sn)

Migrants	*Background electrolyte*	*Remarks*	*Ref.*
Sb, Sn, As, Cd	0.05 *M* citric acid, pH 5.5	Also considered many other ions of groups 2, 3, 4 and 7	18
Sb–Sn	1 *N* HCl	Also separated As from Cu and Hg(II), and Au, Pd, Pt	68
As, Sb, Sn	Many	Separated in pairs. All three ions could not be separated in one run	71
Sb(V), Sn(II), Al(III), Cr(III)	0.1 *M* ammonium tartrate–4 *N* NH_4OH	Complete separation in 4 h at 4 V/cm	54
As(III), Sb(III), Sn(II)	0.1 *M* triethanolamine–0.1 *M* ammonium tartrate		25
As, Sb, Sn, Ag, Pb, Cu, Bi, Cd, Zn, Co, Ni	Many	Separation of binary and ternary mixtures of these ions	50
As, Sb, Sn	0.02 *M* lactic acid–0.02 *M* tartaric acid–0.04 *M* alanine, and others	Discontinuous and continuous electrochromatography	62
As, Sb, Sn		Discontinuous electrochromatography	28
As(V), Sb(III)	0.007 *M* citric acid–5 *N* NH_4OH, and others	Also considered ions of groups 1, 3 and 4	22
As, Sb, Pt, Pd, Ru, Au	Many	Chiefly a separation of As and Sb from the rest. It is claimed here that Sn cannot be separated from the Pt metals by electromigration because of chemical interaction	43
As, Sb, Sn, Al, Fe(III), CrO_4^{2-}	$Na_4P_2O_7$–HCl, pH 2.4		25

This group of ions has been fairly well studied. For some work involving the separation of the oxyanions of these ions (AsO_4^{3-}, for example) from other common anions, see Section 9.

GARRISON *et al.* separated radioactive arsenic in carrier-free form from a $Cu(OH)_2$ precipitate by electromigration in a stack of filter-paper discs[81]. MAKI has separated

Sb(III) from Ni, Cu, and Fe(III), using solutions of complex phosphates as background electrolytes[27]. CETINI[66] separated Sn(II) from some ions of groups 3 and 4 in a starch gel, with NaOH–potassium biphthalate and NaOH–lactic acid electrolyte.

6. THE PLATINUM METALS (Ru, Rh, Pd, Os, Ir, Pt) AND Au

TABLE 6

THE PLATINUM METALS (Ru, Rh, Pd, Os, Ir, Pt) AND Au

Migrants	*Background electrolyte*	*Remarks*	*Ref.*
Pt(IV), Pd(II), Rh(III), Ir(IV)	EDTA solutions of various pH values	In 0.1 *M* EDTA at pH 9, complete separation of all 4 in 45 min at 15 V/cm. Rh(III) precipitated at starting point; Pd, Pt move in that order toward anode	82, 83
Pt(IV)–Pd(II); Pd(II)–Ir(IV); Rh(III)–Ir(IV); Rh(III)–Pt(IV); Rh(III)–Pd(II)–Ir(IV)	Various EDTA solutions	Continuous electrochromatography. Separations as shown	82, 83
Pd(II), Pt(IV), Ir(IV), Rh(III), Ru(III), Os(IV), Au(III)	Several	Separations of quaternary mixtures studied	84, 85
Pt, Pd, and ions of groups 3 and 4	Many	HCl and sodium citrate best electrolytes. Separations of binary mixtures	63
Ru(III), Pt, Pd, and ions of groups 3 and 4	Many	Ru(III) separated from Pt and Pd in KCN, $NaNO_2$ and HCl. Separation of Ru(III) from binary mixtures with other ions also given	64
Pt, Pd, Ru, Au, and some ions of groups 3 and 4	Many	Au separated from Pt in KSCN and oxalic acid, from Pd in KSCN, and from Ru in KCl and KSCN. Separations of Au from some ions of groups 3 and 4 also given	65
Os(IV) and copper group ions	Many	Separation of Os(IV) from the others	43
Pt, Pd, UO_2, Fe(III)		Separation of UO_2 from the others in binary mixtures	43
Pt, Pd, Ru, Au, As, Sb		As, Sb separated from the others in binary mixtures	43
Pd, Au	0.5 *N* NaCl	Separation in 2% agar gel. Present as $PdCl_2$ and $NaAuCl_4$	80

This group has been quite thoroughly investigated by a few workers. Complicated behaviour may occur in some systems—for example, SATO *et al.* report that Ru(III) forms as many as 8 zones in 0.1 *M* lactic acid[29].

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Cu has been separated from Au, Pd, and Pt in 1 *N* HCl[68, 69]. It may be noted here that LEDERER claims that Rh–Pt and Rh–Pd cannot be separated reliably by paper electromigration[86].

7. Ti, V, Zr, Nb, Mo, Tc, Ta, W AND Re

TABLE 7

Ti, V, Zr, Nb, Mo, Tc, Ta, W AND Re

Migrants	*Background electrolyte*	*Remarks*	*Ref.*
Ti(IV), Zr(IV), Th(IV), Fe(III), Al(III), Cr(III), UO_2(II), Be(II)	Several	Separations of binary, ternary, and a few quaternary mixtures were made. Mobilities of these ions are generally low	42
Ti(IV), Zr(IV), Ta(V), Nb(V), Pa(V), Fe(III), Bi(III), Pb(IV), Th(IV), Po(III), U(VI)	HF, and HF–HCl mixtures of various concentration	Zr–Pa–Fe; Zr–Nb; Zr–Pa–U–Th; and a few other separations are given	39
Ti(IV), Zr(IV), Ta(V), Nb(V), Pa(V), Fe(III), U(VI)	5% HF–5% HCl and 1% HF–1% HCl	Zr–Pa–U, and Zr–Pa–Fe separated	40
Mo(IV), V(V), Ti(III), and elements of groups 1, 2, 3, 4 and 5	0.05 *M* citric acid, pH 2.9. Other pH values also used	Other elements also studied, and some separated from this group	18
Ti, V, Mo	0.5 *N* HCl	Separated as the per-ions	87
Nb–Ta	Citric acid–potassium citrate, pH 3.42. Oxalic acid–ammonium oxalate	In first electrolyte, Nb faster. Two zones are formed by Ta in the second electrolyte; one coincides with Nb zone. This is a detailed study	88
W–Mo	Oxalic acid of various concentrations and pH	MoO_4^{2-} faster than WO_4^{2-}	89
Nb–Ta	Sodium borate–NaOH in various proportions	Tantalate much slower than niobate. Separation accomplished in 0.01 *M* NaOH–0.01 *M* $Na_4B_2O_7$ at pH 10.15, or in 0.01 *M* NaOH	89
MoO_4^{2-}, WO_4^{2-}, CrO_4^{2-}, Cr^{3+}	0.1 *M* ammonium citrate, and 0.1 *M* lactic acid	Cr^{3+}–CrO_4^{2-}; CrO_4^{2-}–WO_4^{2-}; CrO_4^{2-}–MoO_4^{2-} separated in ammonium citrate. Lactic acid gave best separation of MoO_4^{2-} and WO_4^{2-}	90
Tc–Re and Tc–Re–Mo	Hydrazine sulfate–hydrazine hydrate, pH 9; 1% $SnCl_2$ in 1 *M* HCl; 0.1 *M* HBr; 1 *M* HI	TcO_4^- and ReO_4^- have similar mobilities. These electrolytes reduce Tc(VII) to Tc(IV) without affecting Re(VII). In first electrolyte, Tc(IV) slow but cationic, ReO_4^- anionic. MoO_4^{2-} also separated here (faster than ReO_4^-). In second electrolyte, Tc cationic, Re streaks as anion. Tc is cationic, Re anionic in the last two	91

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These elements are not easy to work with, but some very good studies have been made of a few systems.

GARRISON *et al.* separated carrier-free Nb from a MnO_2 precipitate, and Nb and Zr from Y and rare earths, by electromigration in a stack of filter-paper discs moistened with 1 *M* ammonium oxalate[81].

8. INORGANIC IONS OF N, P, S, Se AND Te

TABLE 8

INORGANIC IONS OF N, P, S, Se AND Te

Migrants	*Background electrolyte*	*Remarks*	*Ref.*
NH_4^+, $N_2H_6^+$, NH_3OH^+, NO_3^-, NO_2^-, $N_2O_3^{2-}$, $N_2O_2^{2-}$	0.001 *N* H_2SO_4, Na_2SO_4–NaOH, Na_2SO_4–NaOH–$Fe(OH)_3$, Na_2SO_4–Ag_2SO_4	A mixture of all of these could be resolved in 3 runs	92
S^{2-}, $S_2O_3^{2-}$, SO_3^{2-}, SO_4^{2-}	8 *N* NH_4OH		93
$S_3O_6^{2-}$, $S_4O_6^{2-}$, $S_5O_6^{2-}$	1% sodium potassium tartrate	Continuous electrochromatography	60
SeO_3^{2-}–TeO_3^{2-}	0.5 *N* HCl		94
SeO_3^{2-}, SeO_4^{2-}, TeO_3^{2-}, TeO_4^{2-}	0.1 *N* H_2SO_4, and 0.4 *N* Na_2SO_4	Mobilities in Na_2SO_4: $SeO_4^{2-}>SeO_3^{2-}>TeO_3^{2-}>TeO_4^{2-}$. Complete separation in 3 h at 7.5 V/cm in this electrolyte	95
$S_2O_3^{2-}$, and di-, tri-, tetra-, penta-, and hexa-thionate ions	0.05 *M* potassium biphthalate of pH 4.2 and 6.2	Mobility decreases with increasing number of S atoms	96
Thiosulfate, tri-, tetra-, and pentathionates, seleno- and telluro-dithionates, tetrathionates	As above	Se lowers the mobility with respect to the corresponding S compounds; Te lowers it still more	97
Sulfite, thiosulfate, and other photographic reducing agents	Citric acid–phosphate mixture of pH 7		98
SO_4^{2-}–PO_4^{3-}	0.5 *N* HCl	Separation of "carrier-free" radioactive materials	94
$H_2PO_4^-$, HPO_4^{2-}, PO_4^{3-}	Phosphoric acid	A comparison of mobilities	99
Oxyacid anions of phosphorus	0.1 *M* acetic acid, and 0.1 *M* lactic acid	Long (2 m) migration paths. Radioactive species employed. Used also for identification	100

(continued on p. 171)

TABLE 8 (*continued*)

Migrants	*Background electrolyte*	*Remarks*	*Ref.*
Various poly-phosphate ions	Borax–H_3BO_3–NaCl buffer of pH 8.5	Trimeta-, tetrameta-, mono-, di-, tri-, and tetra-phosphates, and others from commercial preparations	101
Various condensed phosphate ions	A number of buffer solutions at various pH values		102
Various condensed phosphate ions	NaOH–borate buffer, and others	A high voltage technique. Mono-, di-, tri-, trimeta-, tetrameta-phosphates, among others, were separated	103
Condensed phosphate ions	Sodium borate–NaOH, pH 10	Continuous electrochromatography	104
Phosphate–silicate	Sodium borate–NaOH, and others	Silicate slower than phosphate	89

Section 9 will deal with phosphates, sulfates, and others when considered in relation to other common anions. It will also cover CN^- and SCN^-.

9. SOME COMMON ANIONS

TABLE 9

SOME COMMON ANIONS

Migrants	*Background electrolyte*	*Remarks*	*Ref.*
PO_3^{3-}, CNS^-, Cl^-, Br^-, and cations	Ethanol–acetic acid–ammonium acetate	Discontinuous electrochromatography. Most of the separation is due to chromatographic effects; electromigration separated only the anions and cations	23
Cl^-, Br^-, I^-	0.1 to 2.5 *M* lactic acid	Br^-, I^- have similar mobilities. All were separated	105
BrO_3^-–Br^-; IO_3^-–I^-; AsO_3^{3-}–AsO_4^{3-}	0.01 *M* NaOH	Separation of products of Szilard-Chalmers reactions	106
PO_3^{3-}, AsO_3^{3-}, AsO_4^{3-}, SO_4^{2-}, SiF_6^{2-}, S^{2-}, SO_3^{2-}, HF_2^-, $S_2O_3^{2-}$, CrO_4^{2-}, $Cr_2O_7^{2-}$	1 *M* formic, acetic, and lactic acids, 8 *M* NH_4OH, and 0.1 and 0.05 *M* ammonium acetate	A number of separations accomplished	93
$Cr_2O_7^{2-}$, PO_4^{3-}, CNS^-, $Fe(CN)_6^{3-}$, $Fe(CN)_6^{4-}$	0.5 *M* lactic acid	$Cr_2O_7^{2-}$ decomposed during migration. Separations in groups of 3 and 4	107
$Fe(CN)_6^{4-}$, $Fe(CN)_6^{3-}$	0.5 *N* HCl	Separation in 50 min at 5 V/cm	94

(continued on p. 172)

TABLE 9 (*continued*)

Migrants	*Background electrolyte*	*Remarks*	*Ref.*
$Fe(CN)_6^{4-}$–$Fe(CN)_6^{3-}$	0.1 *N* KCl		46
PO_4^{3-}–SO_4^{2-}	0.5 *N* HCl	Separation of "carrier-free" radioactive material	94
Phosphate–silicate	Sodium borate–NaOH, and others	Silicate slow	89
ClO_3^-–BrO_3^-–IO_3^-; ClO^-–ClO_2^-–ClO_3^-		Separation of first 3 in 50 min, second 3 in 30 min, at 40 V/cm Mobilities: $IO_3^- > BrO_3^- > ClO_3^-$; $ClO_2^- > ClO_3^- > ClO^-$	108

Since complex formation with the background electrolyte does not take place with anions as it does for many of the metal cations, the choice of the background electrolyte should not have as great an importance as with the latter.

Besides the separations tabulated above, the work giving relative mobilities of many anions should be pointed out again[9–12]. This indicates a number of separations which are possible, and gives a good deal of information about the behaviour of these ions.

Some metal anions such as CrO_4^{2-} also appear in references given in their respective sections when no separations from non-metal anions are involved. Phosphates are treated here in relation to other common anions, but separation of various phosphate ions from one another is covered in Section 8.

MACH[30] used electromigration in paper to check the radiochemical purity of solutions containing radioactive P, S, Cl, and I. CETINI[66] has studied $Fe(CN)_6^{3-}$, $Fe(CN)_6^{4-}$, Cl^-, Br^-, I^-, IO_3^-, AsO_4^{3-}, AsO_3^{3-}, S^{2-}, and SCN^- in a starch gel with ammonium acetate as background electrolyte. LEDERER AND COOK[80] separated $Fe(CN)_6^{3-}$–$Fe(CN)_6^{4-}$, I^-–SCN^-, I^-–CrO_4^{2-}, and SCN^-–CrO_4^{2-} in a 2% agar gel with 1 *N* NaCl as background electrolyte.

10. SEPARATION OF METAL COMPLEX IONS

A number of workers have used electromigration in paper to separate various complex ions of some metals, and others have used it to investigate the behaviour of a metal ion in complex-forming systems. The latter consider for the most part the direction of migration and the mobility under various conditions, and are able to determine the sign of the charge on the complex, as well as something about the variation of its stability under different circumstances. However, since this does not include separations, it will not be dealt with here in any detail.

KAWAMURA *et al.*[109–111] have studied the qualitative and quantitative separation of chromium complexes by electromigration in paper. Separations

of $[Cr(H_2O_6)]^{3+}$, $[Cr(H_2O)_4(C_2O_4)]^+$, $[Cr(H_2O)_3(C_2O_4)(OAc)]$, $[Cr(H_2O_2)_2(C_2O_4)_2]^-$, $[Cr(C_2O_4)_3]^{3-}$, and similar species with the ligands SO_4^{2-}, SCN^-, and NH_3, were made. A good separation was achieved in 0.33 *N* $HClO_4$ in 1 h at 13 V/cm. Distances moved were proportional to the charge on the ion.

MAKI has separated some complexes of Cr(III) in an HCl–KCl electrolyte[112]. At 4 V/cm, 2.5 h were required to separate $[Cr(H_2O)_6]^{3+}$, $[Cr(H_2O)_5Cl]^{2+}$, and $[Cr(H_2O)_4Cl_2]^+$. Under similar conditions, $Cr_2(SO_4)_3$ can be separated into the species $[Cr(H_2O)_6]^{3+}$ and $[Cr(SO_4)]^+$.

SHUTTLEWORTH has investigated sulfate complexes of Cr(III) by electromigration in 0.05 *N* HNO_3[113], while KERTES AND LEDERER have studied these complexes using ^{35}S as tracer[114]. The kinetics of complex formation were followed in this way.

BIGHI AND TRABANELLI[115] separated the complex ions $[Co(NH_3)_6]^{3+}$, $[Co(NH_3)_6Cl]^{2+}$, $[Co(NH_3)_4(NO_2)_2]^+$, and $[Co(NH_3)_3(NO_2)_3]$, as well as similar Cr(III) species, by continuous electrochromatography in 0.25% NH_4Cl. MAKI[116,117] has separated the ions $[Co(NH_3)_6]^{3+}$, $[Co(NH_3)_5NO_2]^{2+}$, *cis* and *trans* $[Co(NH_3)_4NO_2]^+$, $[Co(NH_3)_3(NO_2)_3]$, $[Co(NH_3)_2(NO_2)_4]^-$, and $[Co(NO_2)_6]^{3-}$ by a discontinuous strip technique in a chloride solution.

RAUSCHER AND HARBOTTLE[118] separated the complex species $[Co(CN)_6]^{3-}$, $[Co(CN)_5H_2O]^{2-}$, $[Co(CN)_4(H_2O)_2]^-$, and $[Co(CN)_4(NH_3)_2]^-$, as well as Co^{2+}, in an acetate buffer. These were products of the Szilard-Chalmers reaction in potassium cobalticyanide, and the method was used as an aid in identifying the species as well as estimating their relative amounts.

SHUKLA[124,127] and SHUKLA AND LEDERER[125,126], by electromigration, have studied rhodium(III) as perchlorate, oxalate, and sulfate complexes.

II. SEPARATIONS OF PARTICULAR RADIOCHEMICAL INTEREST

A number of separations have been made which are of particular interest in one or another branch of radiochemistry. The earliest of these is the separation of "carrier-free" activities from precipitates of other elements in a stack of filter-paper discs moistened with a suitable electrolyte, made by GARRISON *et al.*[81]. Elements separated include Nb from MnO_2, and Nb and Zr from Y and the rare earths in ammonium oxalate solution, and As from $Cu(OH)_2$ in HCl. Other separations of interest include that of Ra D, E, and F by SATO *et al.*[26,29], the separation of "carrier-free" $^{32}PO_4^{3-}$ and $^{35}SO_4^{2-}$ from one another by LEDERER[94], and the separation of ^{45}Ca and $^{32}PO_4^{3-}$ from each other and from other radioactive contaminants, by continuous electrochromatography by SATO *et al.*[119]. SCHUMACHER has also used his focussing technique to separate "carrier-free" radioactive materials[5].

Separations of the products of Szilard-Chalmers reactions in potassium cobalticyanide have been made by RAUSCHER AND HARBOTTLE[118], and these separations, as well as those of the products of similar reactions in alkali bromates and iodates, and in arsenic pentoxide, have been described by JACH, KAWAHARA, AND HARBOTTLE[106]. SATO *et al.* have done analogous work with phosphates[120].

References p. 174/176.

MACH has used electromigration in paper to estimate the radiochemical purity of ^{32}P, ^{35}S, ^{22}Na, ^{42}K, ^{86}Rb, ^{131}I, and ^{90}Sr, and recommends it as a routine method for such purposes[30].

Finally, ZIMAKOV *et al.*[121] have used this method to determine quantitatively all of the ions of Ce, Y, Sr, Zr, Nb, Ru, and Cs in a solution of fission products. By carrying out electromigration in 0.1 *N* HCl, 0.1 *N* NaOH, and 2% $K_4Fe(CN)_6$ electrolyte solutions, and using standardized techniques for measuring various sections of the strips, one can obtain quantitative estimates of each of these substances rapidly.

12. MISCELLANEOUS

The separation of isotopes by electromigration procedures has been studied by a number of workers. This has been treated in a recent review by CHEMLA[122], and will not be discussed further.

MAKI[123] has used the zone length to give a method of quantitatively estimating Ba and Al. With proper standardization, the estimation was accurate to within 10% over the range of 150 to 1500 μg of Ba, and 25 to 50 μg of Al. Others have eluted the separated substances from the strip for analysis by standard methods. SCHUMACHER also has adapted his technique for quantitative analysis[7].

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CHROMATOGRAPHY OF LIPIDS ON SILICIC ACID

(Addendum* to the article on pp. 111–133)

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1. INTRODUCTION

An early report[291] on silicic acid chromatography of methyl oleate and its autoxidation products has been found.

COLLINS[292] has published results that apparently reveal degradation by silicic acid of some naturally occurring, complex phospholipids. Other workers will certainly be stimulated by his suggestions that these phospholipids are phosphate triesters, and that they are broken down to give more familiar phospholipids.

2. THE ADSORBENT

Although the chemical and physical structures of silicic acids are continually being studied[293–299] their influence in chromatography is little understood. However, it seems that there are optimum pore diameters for resolution of mixtures of hydrocarbons[294] and steryl acetates[300]. The two most important attributes of silicic acid for resolving mixtures containing polar lipids are probably large pore diameter (40–100 Å) and high silanol content. If the pH is near neutrality when silica gel is precipitated it yields adsorbent having wider pores than if the pH is low[293–295].

Formic acid, which is adsorbed as its anion on alumina and on metals, is not dissociated when adsorbed on silicic acid. The adsorbed molecules may be hydrogen-bonded not only to the silicic acid, but one to another[298].

Methods for preparing and grading samples of silicic acid containing adsorbed water have been described[301]. Adsorbed water undoubtedly improves resolution of certain mixtures of lipids[302–304] if eluents (particularly alcohols) that would elute the water are absent.

3. ELUENTS

A paper[305] on the separation of glycerides (non-phospholipids) on silicic acid-impregnated glass fibre paper provides a useful supplement to Table I (p. 115).

WARNER AND LANDS[306] have published a method for calculating the composition of the eluent during continuous, convex gradient elution.

* Received May 1961.

References p. 180/181.

4. EXPERIMENTAL CONSIDERATIONS

A detailed description has appeared[307] of the methods (including silicic acid chromatography) adopted in one particular laboratory for examination of a natural lipid mixture. NELSON[308] has designed a useful valve for constant-volume fraction collectors.

In preparative work it is useful to plot elution curves spectrophotometrically, in terms of infrared absorption at a selected frequency[309].

Water greatly facilitates autoxidation of *o*-dihydric phenols adsorbed on silicic acid[310].

DOLE AND MEINERTZ[311] have studied the removal of non-lipids from lipids by liquid–liquid partition in heptane–isopropanol–water.

Recent papers on the haemolytic activity[312] of colloidal silica, and its inhibitory effect on some phosphatases[313], show reasons for caution in using lipids eluted from silicic acid in biochemical systems.

Phospholipids can be separated from neutral lipids in ethereal solution by shaking with silicic acid and centrifuging[314].

Hydrocarbons

Whereas only alkanes and monoenes are eluted by hexane, polyenes are eluted by carbon disulphide[315].

Esters other than steryl esters and glycerides

The 1,4-diyne system in methyl isanate retards elution considerably[316]. A methyl mycolate (a diol) has been eluted with benzene[317].

Long-chain alcohols

Docosanol, eluted by 30 % benzene in hexane, precedes triterpene alcohols (which, in turn, precede sterols)[315].

Quinones

SHUNK *et al.*[318] have eluted the di-O-isopropyl homologue of Coenzyme Q_{10} (II, p. 122; $n = 10$) with 6 % ether in hexane.

Quinone K_x has been identified[319] as 2-methyl-3-solanesyl-1,4-naphthoquinone.

Sterols

Three recent papers[320,321] indicate the order of elution, 24,25-dihydrolanosterol > lanosterol > 4α-methyl-Δ^8-cholestenol > 4α-methyl-Δ^7-cholestenol (methostenol) > cholesterol > Δ^7-cholestenol > 7-dehydrocholesterol.

Glycolipids

Numerous new glycolipids are being discovered through the use of silicic acid chromatography[322–330]: examples are one type (isolated from Tay-Sachs brain) containing hexosamine[328], two (from liver) containing ethanolamine[329], and others (from mycobacteria) containing methyl ethers of rhamnose and fucose[330].

Galactosyl monoglycerides are eluted by 10 % methanol in ether[331–333].

References p. 180/181.

Lipoamino-acids

Phosphatidylserines may tend to be eluted later than phosphatidylethanolamines[334,335]; they are eluted much later from silicic acid impregnated with ammonia[336], presumably because of salt formation.

N-Palmityl-DL-phenylalanine is eluted by 50 % ether in hexane[337].

There are more reports[324–326,335,337,338] of uncharacterized lipoamino-acids in eluates from silicic acid.

Glycerophosphatidic acids

L-α-(Dipalmityl)-glycerophosphatidic acid (IV, p. 124; $R_1 = R_2 = (CH_2)_{14}\cdot CH_3$, $R_3 = H$) has been eluted with a continuous gradient of methanol in benzene–chloroform[339], lysophosphatidic acid with 40 % methanol in chloroform[340], and cardiolipin by 5 % methanol in chloroform[341].

Lecithins

Whereas L-α-(diheptanoyl)-lecithin and higher homologues can be eluted with chloroform–methanol mixtures, L-α-(diacetyl)- and L-α-(dibutyryl)-lecithin require methanol–water[342].

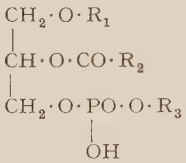
Recent studies indicate[343,344] that lecithins contain unsaturated fatty acid residues exclusively on the 2-position. Thus, the reason why the lecithins that are eluted first from silicic acid are the most unsaturated[341,345,346] is that they contain very unsaturated residues (*e.g.* arachidonate) on the 2-position.

Lysolecithins

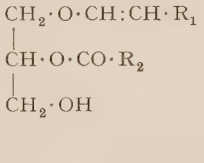
It has been confirmed[342,347] that silicic acid catalyzes rearrangement of 2- to 1-(acyl)-lysolecithins.

Ethers

Apparently the respective saturated ether analogues of phosphatidylethanolamines (VII, $R_3 = CH_2\cdot CH_2\cdot NH_2$) and lecithins (VII, $R_3 = CH_2\cdot CH_2\cdot N^+(CH_3)_3{}^-OH$) are eluted with these phospholipids; the lyso-compounds are eluted late[348].



(VII)



(VIII)

Plasmalogenic diglycerides (VIII) can be eluted before diglycerides[349]. According to HARRIS *et al.*[346], choline plasmalogens tend to be eluted before lecithins.

References p. 180/181.

Miscellaneous lipids

BAER and his co-workers[350,351] have purified numerous synthetic intermediate compounds on silicic acid columns.

Metal-free porphyrins have been eluted with chloroform[352], cyclic polypeptides with chloroform–acetone mixtures[353,354], and α-lipoic acid and its sulphoxide with 1 % methanol in chloroform[355].

N,N-Dimethyl-phosphatidylethanolamines are eluted more slowly than phosphatidylethanolamines[356]. Fatty acyl esters of choline chloride are eluted between lecithins and lysolecithins[342].

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